

Molecular Regulation of β -Lactam Biosynthesis in Filamentous Fungi

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INTRODUCTION

The discovery of antibiotics for clinical use started with the discovery of the efficacy of a β -lactam compound and is perhaps the most important discovery in the history of therapeutic medicine. The application of antibiotics to the therapy of infectious diseases may conceivably have saved more lives than any other medical development (135). It began in 1929, when Alexander Fleming published his observation about the inhibition of growth of *Staphylococcus aureus* on an agar plate contaminated with *Penicillium notatum* (117). Three years later, it was shown that the growth inhibition was due to pen-

icillin (71). The first clinical trials with penicillin were undertaken in 1941 (reviewed in reference 2). In parallel with efforts to provide penicillin in large amounts, its structure was elucidated in 1945, when Hodgkin and Low showed by X-ray crystallography analysis that it is composed of a β -lactam structure (reviewed in reference 1).

During the late 1940s, the fungus *Cephalosporium acremonium* (now renamed *Acremonium chrysogenum*) was isolated from the sea at Cagliari, Italy, by Guiseppe Brotzu (51). This fungus was first found to produce penicillin N; later, another antibiotic was discovered in the culture broth, which was found to consist of different derivatives of a β -lactam compound designated cephalosporin (reviewed in reference 2). The structure of cephalosporin C was described in 1961 by Abraham and Newton (3) and confirmed by X-ray crystallography analysis (141). The discovery of cephalosporin C generated a whole new group of clinically significant β -lactams.

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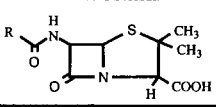
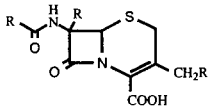
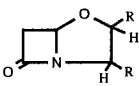
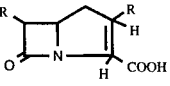
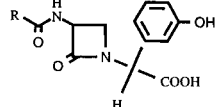
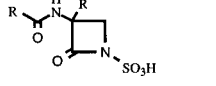
Classes of naturally occurring β -lactams	Antibiotics	Producing microorganisms (Examples)	
		Fungi	Bacteria
Penam 	Penicillins	<i>Penicillium chrysogenum</i> <i>P. notatum</i> <i>Aspergillus nidulans</i>	Gram ⁺
Ceph-3-em 			Gram ⁻
Clavam 	Clavulanic acid		<i>Streptomyces clavuligerus</i>
Carbapenem 	Thienamycins Olivanic acid Epithienamycins		<i>Streptomyces clavuligerus</i> <i>S. olivaceus</i> <i>Erwinia carotovora</i> <i>Serratia</i> sp.
Monolactam 	Nocardicines		<i>Nocardia uniformis</i> subsp. <i>tsuyamanensis</i>
	Monobactams		<i>Agrobacterium radiobacter</i> <i>Pseudomonas acidophila</i>

FIG. 1. Naturally occurring classes of β -lactam antibiotics essentially as compiled by O'Sullivan and Sykes (249). Modified from reference 8 with permission of the publisher.

The success of β -lactams in the treatment of infectious disease is due to their high specificity and their low toxicity. Despite a growing number of antibiotics and the incidence of penicillin-resistant isolates, β -lactams are still by far the most frequently used antibiotics (299).

However, it is only in the past 20 years that the biosynthetic pathways leading to penicillins (penams) and cephalosporins (ceph-3-em)s have been elucidated. This is partly because biosynthetic enzymes are often unstable and are present in the cell in only small amounts, making their purification difficult. In addition, industrial production of penicillin and cephalosporin was achieved with *P. chrysogenum* and *A. chrysogenum*, respectively. These fungi, however, belong to the deuteromycetes, which are in general difficult to analyze genetically. Currently, the greatest progress in elucidation of the molecular regulation of biosyntheses of β -lactams in fungi has been made in the penicillin producer *Aspergillus (Emericella) nidulans*, since this fungus is an ascomycete with a sexual cycle. Therefore, classical genetic techniques can be applied to *A. nidulans* (262) and hence a detailed genetic map is available (70). Together with molecular techniques, this facilitated a thorough analysis of the genetic regulation of metabolic pathways, including that of penicillin biosynthesis (reviewed in references 20, 49, and 204).

Since the biochemistry of penicillin and cephalosporin biosynthesis is rather well understood and recombinant techniques have been developed for some filamentous fungi, recent research has aimed at elucidating the molecular regulation of

β -lactam biosynthesis. Within the last few years, several studies have indicated that the β -lactam biosynthesis genes are controlled by a complex regulatory network. A comparison with known regulatory proteins and DNA elements of eukaryotes involved in the regulation of genes of primary metabolism is thus of great interest. Such investigations might also provide hints about both the evolution of secondary metabolism and the signals leading to the production of β -lactams. Furthermore, the overexpression of regulatory genes will lead to higher yields of β -lactams in the respective production strains and knowledge of biosynthesis genes will allow the production of new compounds by combinatorial biology.

The biosynthesis of β -lactam compounds and their molecular genetics were the subject of several recent reviews (8, 45, 49, 152, 211, 299). In particular, the regulation of β -lactam biosynthesis in fungi has seen a tremendous increase in knowledge within the last years, and it is this aspect which is considered in most of the present review.

GENERAL ASPECTS OF β -LACTAM COMPOUNDS

β -Lactams can be classified into five groups on the basis of their chemical structures (Fig. 1). All of these compounds have in common the four-membered β -lactam ring. Apart from the monolactams, which have a single ring only, β -lactams consist of a bicyclic ring system. The ability to synthesize β -lactams is widespread in nature. It has been found in some fungi but also

in some gram-positive and gram-negative bacteria (Fig. 1). However, although organisms belonging to all three groups can synthesize the hydrophilic cephalosporin compounds (ceph-3-ems), the hydrophobic penicillins are produced as end products only by filamentous fungi (Fig. 1). For the remaining groups of β -lactams listed in Fig. 1, only bacterial producers have been reported so far. The number of prokaryotic and eukaryotic microorganisms recognized as being able to synthesize β -lactam antibiotics is continuously increasing (152).

BIOSYNTHESIS OF PENICILLINS AND CEPHALOSPORINS: AN OUTLINE

To give a brief overview of the complete biosynthetic pathways, references for fungal enzymes and genes have been omitted in the text and are given in the tables and the following sections.

The biosyntheses of penicillins and cephalosporins have the first two steps in common (Fig. 2). All naturally occurring penicillins and cephalosporins are formed from the same three amino acids: L- α -amino adipic acid (L- α -AAA), L-cysteine, and L-valine. L- α -AAA is a nonproteinogenic amino acid and is derived from the fungus-specific aminoadipate pathway which leads to the formation of L-lysine. It can also be provided, at least in *A. nidulans* and *P. chrysogenum*, by catabolic degradation of L-lysine, although the contribution of this pathway to penicillin biosynthesis in these fungi has not been clarified yet (see "Amino acids as precursors and mediators of regulation," below).

In the first reaction cycle, the amino acid precursors are condensed to the tripeptide δ -(L- α -amino adipyl)-L-cysteinyl-D-valine (ACV). All reactions which are required for the formation of this tripeptide, e.g., specific recognition of amino acids, their activation via the formation of aminoacyl adenylates, and formation of peptide bonds, are catalyzed by a single multifunctional enzyme designated according to the product formed, ACV synthetase (ACVS) (Fig. 2). ACVS is encoded by a single structural gene designated *acvA* (*pcbAB*) (Fig. 2; Table 1).

In the second step, oxidative ring closure of the linear tripeptide leads to the formation of a bicyclic ring structure, i.e., the four-membered β -lactam ring fused to the five-membered thiazolidine ring, which is characteristic of all penicillins. The resulting compound, isopenicillin N (IPN), possesses weak antibiotic activity and is thus the first bioactive intermediate of both the penicillin and cephalosporin pathways. This reaction is catalyzed by isopenicillin N synthase (IPNS), encoded by the *ipnA* (*pcbC*) gene (Table 2). IPN is the branch point of the penicillin and cephalosporin biosyntheses.

In the third and final step of penicillin biosynthesis, the hydrophilic L- α -AAA side chain of IPN is exchanged for a hydrophobic acyl group; the exchange is catalyzed by acyl co-enzyme A (CoA):isopenicillin N acyltransferase (IAT). The corresponding gene has been designated *aatA* (*penDE*) (Table 3). In their natural habitats, penicillins DF, F, and K, which contain hexenoic acid, Δ^3 -hexenoic acid, and octenoic acid as side chains, respectively, are synthesized. By supplying the cultivation medium with phenoxyacetic or phenylacetic acid, the synthesis can be directed mainly toward penicillin V or penicillin G, respectively (Fig. 2, shown for penicillin G). The side chain precursors must be activated before they become substrates for the IAT. It is generally believed that the activated forms of the side chains consist of their CoA thioesters, but the mechanism behind this activation has still not been fully elucidated.

The first step that commits the pathway to the production of

cephalosporins is the isomerization of the L- α -AAA side chain of IPN to the D enantiomer to give penicillin N. This reaction is catalyzed by IPN epimerase (Fig. 2). Penicillin N is the precursor of antibiotics containing the ceph-3-em nucleus, i.e., cephalosporins and cephamycins (7-methoxycephalosporins), produced by fungi and bacteria, respectively (Fig. 1 and 2). Penicillin N is converted to deacetoxycephalosporin C (DAOC) by DAOC synthetase (expandase) activity (Fig. 2). This ring expansion step involves the oxidative opening of the penam thiazolidine ring to give, upon ring closure, the six-membered dihydrothiazine ring, which is characteristic of all ceph-3-ems. In the next step, the methyl group at C-3 of DAOC is hydroxylated and oxidized to form deacetylcephalosporin C (DAC) (Fig. 2). In *A. chrysogenum*, both reactions are catalyzed by a single enzyme, DAOC synthetase (expandase)/DAC hydroxylase, encoded by the *cefEF* gene, whereas in the bacterial cephalosporin producer *Streptomyces clavuligerus*, one enzyme has been found for each reaction, encoded by the two genes *cefE* and *cefF* (Table 4).

In the last step of cephalosporin C biosynthesis, which is best studied in the fungus *A. chrysogenum*, an acetyl moiety from acetyl-CoA is transferred to the -OH group of DAC; this step is catalyzed by the product of *cefG*, acetyl CoA:DAC acetyltransferase (Fig. 2; Table 5). Several cephalosporins that differ from cephalosporin C in the substituent attached to the 3' C oxygen have been isolated from a variety of microorganisms (152).

In addition, cephalosporins carrying a methoxy group at C-7 (7-methoxycephalosporin, or cephamycin) are produced by both *S. clavuligerus* (compound A-16884A [138]) and *S. lipmanii* (239, 265) (Fig. 2). The specific reactions leading to the formation of cephamycin C, which have been studied best in *S. clavuligerus*, start from the intermediate DAC (Fig. 2). A carbamoyl group is attached to DAC to give O-carbamoyl-DAC (OCDAC). This reaction is catalyzed by 3-hydroxymethyl ceph-3-em O-carbamoyltransferase, which is encoded by the *cmcH* gene (79) (Fig. 2). Then, C-7 is hydroxylated by the action of OCDAC hydroxylase, encoded by *cmcI* (343) (Fig. 2). In the final step of cephamycin biosynthesis, the hydroxy group at C-7 is methylated to form cephamycin C (7-methoxycephalosporin C); the reaction is catalyzed by cephamycin C synthetase, whose corresponding gene has been designated *cmcJ* (79).

GENETIC NOMENCLATURE

Before their identification, the putative genes encoding ACVS were designated *pcbA* (penicillin cephalosporin biosynthesis) and *pcbB*, because it was believed that two enzymes were involved in the formation of an L-(α -amino adipyl)-L-cysteine (AC) dipeptide and the final ACV tripeptide, respectively (Fig. 1) (reviewed in references 147 and 245). Cloning and sequencing of the corresponding gene revealed, however, that a single polypeptide encoded by a single gene is responsible for the formation of the ACV tripeptide. Publications reporting the DNA sequence of the *P. chrysogenum*, *A. nidulans*, and *A. chrysogenum* genes named the gene *acvA*, which reflected the involvement of one genetic locus in the synthesis of ACVS (201, 203, 305, 307), or *pcbAB*, derived from the combination of *pcbA* and *pcbB* (91, 130). In the following discussion, the term *acvA* is used because it indicates that a single gene encodes ACVS. The same is relevant for the gene encoding IAT, which has been named *penDE* or *aat*. In this review, the gene is designated *aatA*, reflecting both the correct genetic nomenclature and the fact that one genetic locus encodes the enzyme. The IPNS gene has been named *ipnA*. The

alternative names are shown in parentheses at the beginning of the relevant sections.

CLUSTERING OF BIOSYNTHESIS GENES

As far as we know, in bacteria and fungi all structural genes of β -lactam biosyntheses are clustered (Fig. 3). In fungi, first the penicillin biosynthesis genes of both *A. nidulans* and *P. chrysogenum* are tightly clustered (201, 305). In the following, in *A. chrysogenum*, two clusters containing the cephalosporin biosynthesis genes have been identified (130, 131, 219, 220), whereas in cephamycin C-producing bacteria, the genes are organized into a single cluster (Fig. 3).

The linkage of antibiotic biosynthesis genes is a well-known phenomenon in many antibiotic-producing organisms. It has been speculated that linkage occurred during evolution owing to an ecological selective advantage (212). Seno and Baltz (294) suggested that coordinated regulation of antibiotic biosynthesis genes could be achieved by organizing the genes into large operons controlled by a single promoter. For example, genes of the actinorhodin biosynthetic pathway in *Streptomyces coelicolor* are clustered and expressed in several polycistronic mRNAs (207). In eukaryotic fungi, however, β -lactam biosynthesis genes are transcribed separately and are expressed from different promoters (reviewed in reference 49). Hence, in fungi, there is no obvious need for clustering, and it thus seems more likely that linkage reflects a common ancestral origin (see "Origin of β -lactam biosynthesis genes in fungi"). However, there is no evidence that the IAT gene (*aatA*) has a close relative in modern prokaryotes, even though it is part of the cluster. This fact supports the hypothesis that linkage might also confer an ecological advantage to the eukaryotic fungi in their natural habitat, although the reason for this is not yet understood.

STRUCTURAL GENES AND DEDUCED PROTEINS

Genes Common to Penicillin- and Cephalosporin-Producing Fungi

acvA (*pcbAB*), encoding ACVS. The first reaction of the cephalosporin and penicillin biosynthesis is the formation of the ACV tripeptide, which was first found in 1960 as an intracellular component of *P. chrysogenum* (16). All the reactions required for synthesis of the tripeptide are catalyzed by a single enzyme, ACVS, which is encoded by the *acvA* (*pcbAB*) gene (reviewed in reference 169) (Fig. 2; Table 1). Thus, the ACV tripeptide is formed via a nonribosomal enzyme thiotemplate mechanism from its amino acid precursors. This is similar in many aspects to the synthesis of other microbial peptides (reviewed in reference 169).

Early studies with cell-free systems of *P. chrysogenum* (185) and *A. chrysogenum* (30) demonstrated the existence of ACVS activity. It was suggested that two separate enzymes may be involved in the formation of ACV. However, based on the observation that less ACV is formed from the dipeptide AC and L-valine than from the free amino acids, Banko et al. (31) proposed that a single multifunctional enzyme may be responsible for ACV synthesis. This proposal was supported by the first isolation of an ACVS protein by van Liempt et al. (333), who purified ACVS of *A. nidulans* 118-fold. Their results suggested that ACVS consists of a single polypeptide chain. Since then, ACVS enzymes have been purified from different organisms, including *S. clavuligerus* (25, 26, 157), *A. chrysogenum* (25, 26, 345), and *Nocardia lactamdurans* (74). Attempts to purify ACVS from *P. chrysogenum* have thus far been unsuccessful

because the enzyme seems to be rapidly degraded during chromatographic purification (7).

Although not entirely clarified, it is believed that ACVS multienzymes are monomers with molecular masses of around 420 kDa and exhibit different catalytic activities, such as the specific recognition of the three amino acid precursors and their activation, peptide bond formation, isomerization of the L-valine moiety to the D form, and release of the peptide. As in ribosomal peptide biosynthesis, the carboxyl function of the amino acid is activated by the formation of a mixed anhydride with the α -phosphate of ATP, resulting in the release of pyrophosphate (PP_i). This has been used to develop assays based on amino acid-dependent exchange of ³²P between PP_i and ATP (333).

After activation of an amino acid, the aminoacyl adenylate formed is cleaved by the action of an enzyme thiol, resulting in the formation of a thioester bond between the enzyme (at an appropriate location on the enzyme) and the amino acid and in the release of AMP. These thioesterified amino acids play the same role as the tRNA-bound amino acids in the ribosomal peptide biosynthesis. They are high-energy intermediates, which are the targets for nucleophilic attack by the amino group of a second amino acid, resulting in the formation of a peptide bond. As in the ribosome, the nascent peptide grows from the amino terminus to the carboxy terminus and the intermediate peptides remain bound (as thioesters) to the enzyme (169). The substrate specificity is less strict than in protein synthesis, since a variety of tripeptide analogs are known (28).

By assuming three independent activation sites, the dissociation constants for the *S. clavuligerus* ACVS have been estimated to be 1.25 and 1.5 mM for cysteine and ATP, respectively, and 2.4 and 0.25 mM for valine and ATP, respectively. No L- α -AAA-dependent ATP-PP_i exchange was detected with the enzyme preparation used, although the amino acid binding to the enzyme was dependent on ATP (292). The reason for the lack of detection of L- α -AAA-dependent ATP-PP_i exchange remains obscure, because fungal ACVS (from both *A. nidulans* and *A. chrysogenum*) drove radioactivity exchange that was dependent on all three amino acids (292, 333). Dissociation constants for aminoacyl-tRNA synthetases are much lower than those of *S. clavuligerus* ACVS, usually below 100 μ M for their respective amino acids (64, 150, 161). This may be a way of guaranteeing the supply of amino acids to the primary metabolism and avoiding the depletion of vital cellular components by secondary metabolism (292). L-Valine is apparently epimerized to the D form at the tripeptide stage, since no D-valine intermediate has been detected (292).

Each ACVS is encoded by a single structural gene (designated *acvA*) of more than 11 kb (Table 1). The clustering of penicillin biosynthesis genes (Fig. 3) was first shown for *A. nidulans* (201, 305) and *P. chrysogenum* (305). The identification of the gene cluster was based on the assumption that biosynthesis genes for antibiotics are clustered, and information had accumulated about *ipnA* genes from several organisms. Hence, by cross-hybridization with *ipnA* genes as the probe, the *acvA* genes from both organisms were detected upstream from the *ipnA* genes, separated by about 1 kb (Fig. 3). In *A. nidulans*, the presence of an open reading frame (ORF) upstream of the *ipnA* gene was confirmed by disruption of the upstream region, which led to a non-penicillin-producing phenotype of the transformants (201, 305). Furthermore, MacCabe et al. (201) had purified *A. nidulans* ACVS and used it to generate partial amino acid sequence data, from which oligonucleotides were deduced and synthesized. They were used for Southern analysis, which showed that the ACVS-

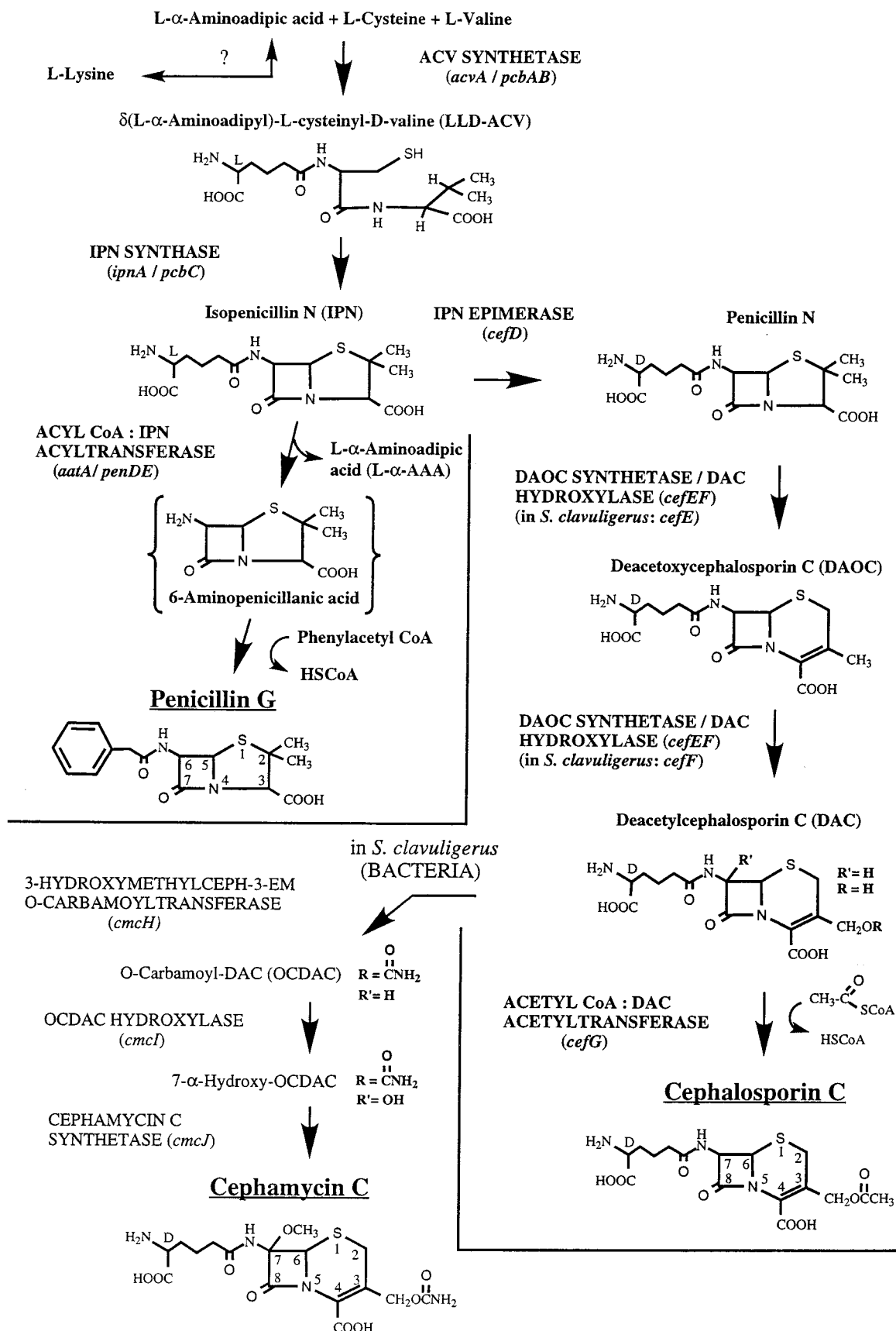


FIG. 2. Biosynthesis of penicillin, cephalosporin C, and cephameycin C. Gene and organism names are in italics, names of enzymes are in capital letters. L- α -AAA is an intermediate of the L-lysine biosynthetic pathway but can also be provided by catabolic degradation of L-lysine. The question mark indicates that the contribution of the latter to β -lactam production has not been clarified yet. The penicillin biosynthesis occurs in fungi only, whereas cephalosporins are synthesized in both fungi (e.g., cephalosporin C by *A. chrysogenum*) and bacteria (e.g., cephameycin C by *S. clavuligerus*) (Fig. 1). See the text for details.

TABLE 1. *acvA* (*pcbAB*) genes of the different fungi encoding ACVS

Characteristic	<i>A. nidulans</i>	<i>P. chrysogenum</i> strains		<i>A. chrysogenum</i>
		Oli13	AS-P-78	
Genome DNA (bp)	11,310	11,328 ^b	11,376	11,136
No. of amino acids	3,770	3,776 ^b	3,792	3,712
<i>M_r</i>	422,486	423,996 ^b	425,971	414,791
Transcript size (kb)	>9.5	ND ^a	11.5	11.4
Transcript start position	Major, -230; minor, -317, -195, -188	ND	ND	ND
No. of introns	0	0	0	0
No. of domains	3	3	3	3
Reference(s)	201, 203	305, 307	91	130

^a ND, not determined.^b Correction of published sequence in reference 49. Translation start moved upstream by 90 bp. The discrepancy between published sequences of *P. chrysogenum* is now at the 3' end.

encoding gene is in fact upstream of the *ipnA* gene. In addition, the molecular mapping data obtained predicted a size of more than 11 kb for the *acvA* gene (201, 305). This was supported by MacCabe et al. (201), who first showed by Northern blot analysis that the *acvA* transcript is indeed larger than 9.5 kb. This finding was confirmed by the sequencing of the *acvA* genes of *A. nidulans* (203), *P. chrysogenum* (91, 307) and *A. chrysogenum* (130) and by a Northern blot analysis of the *P. chrysogenum acvA* gene (91) (Table 1). Subsequently, the corresponding *acvA* genes were also cloned and sequenced from bacterial cephamycin producers such as *S. clavuligerus* (Fig. 3).

Even in fungi, the *acvA* genes form a single ORF, which does not seem to be interrupted by introns, although this assumption has not been proved yet by sequencing of the respective cDNAs. The properties of genes and their deduced enzymes are summarized in Table 1. Fungal *acvA* genes are divergently oriented with respect to the *ipnA* genes (Fig. 3). The sizes of the intergenic regions between the genes vary slightly among the different fungi and are about 1 kb long (Fig. 3). In both *A. nidulans* and *P. chrysogenum*, the *acvA* mRNA starts within the intergenic region between *acvA* and *ipnA* (Table 1; Fig. 3). *acvA* expression is driven by a rather weak promoter that is probably located entirely in the intergenic region between *acvA* and *ipnA* (see "Expression of biosynthesis genes under standard fermentation conditions" below).

Amino acid sequences of ACVS proteins of all fungal and bacterial species so far identified contain three homologous regions of about 600 amino acids. These contain repeated domains that have extensive amino acid sequence similarities to each other, to the corresponding regions of the ACVS protein of other fungi and bacteria, and to the repeated domains identified for *Bacillus brevis* gramicidin S synthetases 1 and 2 and tyrocidine synthetase I (169, 329). Since all of these enzymes specifically recognize amino acids and form adeny-

lates, it is most likely that the respective adenylate-forming domains (for the nomenclature of ACVS domains, see reference 169) recognize and adenylate one of the constituent amino acids. The order of the biosynthesis of the δ -(L- α -AAA)-L-Cys-D-Val tripeptide is believed to reflect the linear organization of the ACVS in AAA-, Cys- and Val-activating domains (reviewed in reference 169). A surprising result, however, was the observation of the formation of *O*-methyl-seryl-D,L-valine by ACVS upon replacement of cysteine by *O*-methylserine (297). This finding suggested that the second peptide bond is initially formed. Consequently, an order of peptide formation starting with Cys-Val and continuing with addition of L- α -AAA would thus be conceivable. To get more information about the order of peptide formation, Kallow et al. (162) investigated enzyme-bound intermediates by omitting the last amino acid, L-Val, in an in vitro reaction. Depending on the reaction mode, this would lead to the accumulation of either L-cysteinyl- or L- α -AAA-L-cysteinyl intermediates bound to the *A. nidulans* ACVS enzyme. In fact, the formation of the AC thioester in the absence of L-Val was observed. It was concluded that the first peptide bond is formed between the δ -carboxyl of L- α -AAA and L-Cys and that this is followed by addition of the dipeptidyl intermediate to L-Val. The formation of *O*-methylseryl-D,L-valine by ACVS previously observed (297) was suggested to be due either to a side reaction initiating peptide synthesis in position 2 of ACVS or to an N-terminal cleavage of the N-terminal aminoadipyl side chain of the tripeptide formed (162). This conclusion is also consistent with the result that L-Cys-D-Val is not a substrate for ACV biosynthesis (297) while δ -(L-AAA)-L-Cys is accepted as a substrate for adenylation and biosynthesis (28, 31; reviewed in reference 7).

Using a microbiological assay for detection of pantothenic acid, Baldwin et al. (25, 26) observed that 1 mol of pantothenic acid was liberated per mol of purified *A. chrysogenum* ACVS.

TABLE 2. Fungal *ipnA* (*pcbC*) genes encoding IPNS

Characteristic	<i>A. nidulans</i>	<i>P. chrysogenum</i>	<i>A. chrysogenum</i>
Genomic DNA (bp)	993	993	1,014
No. of amino acids	331	331	338
<i>M_r</i>	37,480	38,012	38,416
Transcript size (kb)	~1.7	1.1	~1.5, 1.15
Transcript start position ^a	Major, -106	-11	Major, -72 (-73), -56 (-51); minor, -78 (-80), -58 (-54), (-97)
No. of introns	0	0	0
References	255, 257, 266, 339	34, 63, 304	129, 283, 303

^a Values for transcript starts were determined by primer extension or S1 mapping (in parentheses).

TABLE 3. *aatA* (*penDE*) genes of different fungi encoding IAT

Characteristic	<i>A. nidulans</i>	<i>P. chrysogenum</i>
Genomic DNA (bp)	1,237	1,274
No. of amino acids	357	357
M_r	39,240	39,943
Transcript size (kb)	ND ^a	1.15
Transcript start position	-61 (-60), -52, -82	ND
No. of introns	3	3
Posttranslationally processed	+	+
References	194, 234, 325, 341	35, 325, 334

^a ND, not determined.

This implied the presence of one phosphopantetheine molecule per ACVS molecule. It was therefore thought that ACVS follows the classical thiotemplate mechanism; i.e., after activation as aminoacyl adenylates, the intermediates bound as thioesters are assembled by one central swinging arm, the cofactor 4'-phosphopantetheine. Sequencing of the ACVS structural genes (Table 1) revealed, however, that in the three repeated regions of each ACVS, some similarity to 4'-phosphopantetheine attachment sites described for polyketide synthases (i.e., Asp-Ser-Leu) is evident (203). This may reflect the attachment of multiple cofactors to ACVS. Because a single phosphopantetheine arm is sufficient for the activity of fatty acid synthases, the finding of several phosphopantetheine attachment sites suggests a modified mechanism for the thiotemplate pathway to polypeptides (multiple-cofactor model) (203, 291, 313, 314). Although the relevance of all three pantetheine attachment sites of ACVS enzymes has not been proved experimentally, it is believed that peptide assembly is accomplished by the transfer of acyl intermediates between adjacent cofactors (313, 314). In the carboxyl-terminal region of the ACVS enzymes, sequence similarities to the thioesterase active-site region, GX SXG, have been found which would be required to release the generated tripeptide from the enzyme (203).

The current view of the thiotemplate mechanism of ACVS catalysis is summarized in detail by Zhang and Demain (347) and by Kleinkauf and von Döhren (169).

ACVS enzymes are especially interesting since they represent a route for peptide bond formation independent of the ribosome and allow the incorporation of many nonproteinogenic amino acids (28, 168). Furthermore, since different parts of peptide synthetases are specific for certain amino acids, this can be used to engineer genetically new peptide synthetases, and hence to produce new compounds, possibly with new pharmacological activities. This approach has been successfully used by Stachelhaus et al. (312) and is summarized in "Applications" below.

TABLE 4. *cefEF* genes encoding DAOC/DAC

Characteristic	<i>A. chrysogenum</i> <i>cefEF</i>	<i>S. clavuligerus</i>	
		<i>cefE</i>	<i>cefF</i>
Genomic DNA (bp)	996	933	954
No. of amino acids	332	311	318
M_r	36,462	34,519	34,584
Transcript size (kb)	1.2		
Transcript start position	ND ^a		
No. of introns	0		
Reference(s)	131, 284	177	175

^a ND, not determined.TABLE 5. *cefG* gene encoding acetyl-CoA:DAC acetyltransferase

Characteristic	<i>A. chrysogenum</i> strains		
	C10	PLC	IS-5
Genomic DNA (bp)	1,332 ^b	1,300 ^b	1,299 ^b
No. of amino acids	444 ^b	399 ^b	385 ^b
M_r	49,269 ^b	41,000 ^b	
Transcript size (kb)	1.4	1.5	
Transcript start position	ND ^a	-71, -114	ND
No. of introns	2	2	2
Reference(s)	131	219	220, 221

^a ND, not determined.^b Differences in the size of the published genes result in part from differences in fixation of the start ATG.

***ipnA* (*pcbC*), encoding IPNS.** The second step of the penicillin and cephalosporin biosynthesis, i.e., cyclization of the linear ACV tripeptide to the bicyclic IPN, is catalyzed by IPNS (cyclase), a nonheme-Fe(II)-dependent oxidase (106, 107, 174, 248, 340) (Fig. 2; Table 2). The enzyme has a molecular mass of about 38 kDa and formally catalyzes the removal of four hydrogen equivalents of the ACV tripeptide in a desaturative ring closure with concomitant reduction of dioxygen to water (reviewed in reference 279).

IPNS activity was first detected in cell extracts of *A. chrysogenum* (107, 174, 248). The IPNS reaction requires ferrous iron, molecular oxygen as the cosubstrate, and ascorbate as the electron donor to form the β -lactam and thiazolidine ring of IPN (151, 288, 340; reviewed in reference 245). It was shown that *P. chrysogenum* IPNS is strongly inhibited by glutathione and is also sensitive to cobalt inhibition (267). IPNS was purified to homogeneity from *A. chrysogenum* (27, 142, 251) and has subsequently been obtained from *P. chrysogenum* (267), *A. nidulans* (339), several actinomycetes such as *S. clavuligerus* (153), and the gram-negative bacterium *Flavobacterium* sp. (250). It was shown that two interconvertible forms of the enzyme exist, an oxidized state with a disulfide linkage and a reduced state (27).

Only the free thiol form of the tripeptide ACV serves as a substrate; the bis-disulfide dimer which is spontaneously formed is inactive (259). *S. clavuligerus* possesses a disulfide reductase that recognizes bis-ACV as a substrate (6). In *P. chrysogenum*, a broad-range disulfide reductase belonging to the thioredoxin family of oxidoreductases was found which efficiently reduced bis-ACV to the thiol monomer. When coupled to IPNS in vitro, it converted bis-ACV to IPN and was therefore suggested to play a role in penicillin biosynthesis (72). In enzyme assays in vitro, the thiol groups of both the ACV tripeptide and the IPNS enzyme are kept in a reduced state by the addition of ascorbate and dithiothreitol (see, e.g., reference 46). In these assays, the appearance of antibiotic activity due to the formation of IPNS from the antibiotically inactive ACV is measured with an indicator organism which is sufficiently sensitive (174). Alternatively, IPN can be monitored by high-pressure liquid chromatography (154).

Mössbauer, electron paramagnetic resonance, and optical spectroscopy suggested that ACV binds directly to the active-site iron of IPNS via the cysteinyl thiol of ACV (65). A six-coordinate metal center at the active site was proposed, with two or three endogenous histidine ligands, an aspartate, and sites for the thiolate of ACV, oxygen, and solvent (232). It was shown that the ACV sulfur atom binds to the active-site iron of the enzyme (247, 270). The crystal structure of the *A. nidulans* IPNS was recently solved at a resolution of 2.5 and 1.3 Å

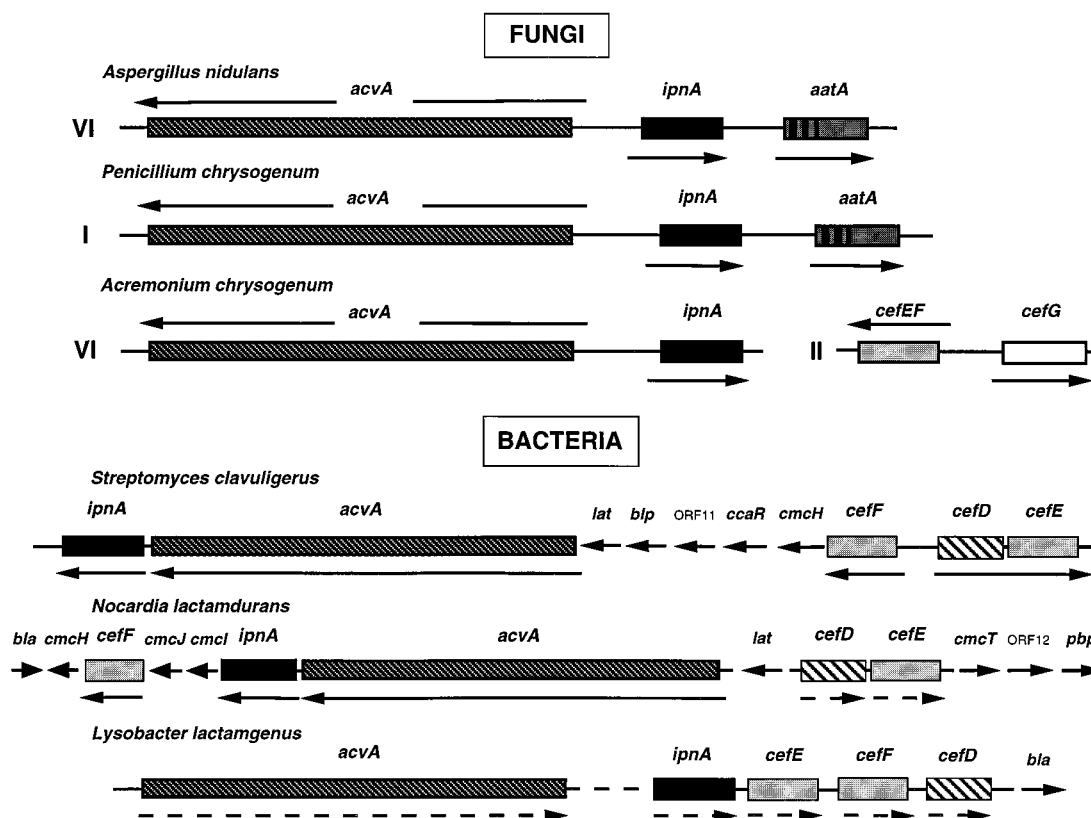


FIG. 3. β -Lactam biosynthesis gene cluster in fungi and bacteria. References to each fungal gene are listed in Tables 1 to 5 or in the text. The *A. chrysogenum cefD* gene has not been identified yet. The whole gene cluster of *N. lactamdurans* has been characterized (76–79). The organization of the *L. lactamgenus* gene cluster was taken from references 166 and 167. Roman numerals indicate the chromosomes (in fungi) on which the genes are localized (115, 201, 235, 300). The intergenic regions between *acvA* and *ipnA* of *P. chrysogenum*, *A. nidulans*, and *A. chrysogenum* are 1,107 bp (91, 307), 872 bp (203) and 1,233 bp (230), respectively. Bacterial genes with fungal homologs are boxed. The transcriptional orientation and the transcript units (Bacteria), as far as it has been determined, are indicated by arrows above or below the boxes. Arrows between boxes (Bacteria) and arrows with broken lines below boxes mark the orientation of genes. The function of ORF is unknown. Abbreviations (76–79, 258): *cmcT*, transmembrane protein; *pbp*, penicillin binding protein; *bla*, β -lactamase; *bfp*, showing similarity to the extracellular β -lactamase inhibitory protein BLIP.

complexed with manganese (279) and with Fe^{2+} and substrate (280), respectively. The secondary structure of IPNS was found to consist of 10 helices and 16 β -strands. Eight of the β -strands fold to give a “jelly-roll” motif. The active-site structure shows the manganese ion attached to four protein ligands (His 214, Asp 216, His 270, and Gln 330) and bears two water molecules occupying coordination sites directed into a hydrophobic cavity within the protein (279). The Fe(II) -ACV-IPNS structure has one protein molecule with ferrous ion and ACV bound at the active site. The side chain of Gln 330, which coordinates the metal in the absence of substrate, is replaced by the ACV thiolate (280). In the substrate complex, three of the five coordination sites are occupied by protein ligands: His 214, His 270, and Asp 216 (43). The remaining two sites are occupied by a water molecule (at position 298) and the ACV thiolate (280). Such a structural characteristic (an iron-binding site within an unreactive hydrophobic substrate-binding cavity) is probably a requirement for this class of enzyme, since it results in the isolation of the reactive complex and subsequent intermediates from the external environment. Thus, the reaction can be channelled along a single path, avoiding the many side reactions potentially open to the highly reactive species resulting from the reduction of dioxygen at the metal (279). The role of enzymes in such processes has been designated negative catalysis (275). IPNS catalyzes a unique enzyme reaction with no precedent in chemistry (279).

All intact IPNS enzymes whose genes have been cloned to date have proline at position 285 in a highly conserved region (269; reviewed in reference 152). This Pro residue seems to be essential for activity because a mutant (N2) of *A. chrysogenum* (298) which did not produce cephalosporin encodes a defective *ipnA* gene, probably due to the mutation of a single base pair that results in a change from Pro (amino acid 285) to Leu (amino acid 269).

Baldwin and Wan (29) proposed a catalytic mechanism for IPNS which involves the formation of an intermediate carbon radical of the LLD form of ACV, but complete details of the reaction have yet to be determined. Additional data on the mechanism of the IPNS reaction suggests that initial formation of the β -lactam ring is followed by closure of the thiazolidine ring (24). A model was proposed by Roach et al. (279, 280).

The IPNS shows a broad substrate specificity, in particular with alterations in the L- α -AAA moiety and the valine residue of ACV. This finding has been ingeniously used to create novel penicillins from ACV analogs in vitro, although cyclization of unnatural tripeptides occurs at lower efficiency (342). Nevertheless, many new penicillins have been produced biosynthetically via this route (23), which is thus very promising for the generation of new β -lactam compounds in vivo.

The genes encoding IPNS enzymes are designated *ipnA* (*pcbC*). The *ipnA* gene from *A. chrysogenum* was the first gene encoding an enzyme of β -lactam biosynthesis to be cloned and

sequenced (283). This was achieved by purification of the enzyme, determination of its N-terminal amino acid sequence, and subsequent cloning of the gene by reverse genetics. The gene was overexpressed in *Escherichia coli*. *ipnA* genes have since been cloned and sequenced from several different fungi and bacteria. Their features are summarized in Table 2.

The *ipnA* and *acvA* genes lie close together on the chromosome. In contrast to bacteria, in fungi *ipnA* and *acvA* are bidirectionally oriented (Fig. 3). For the fungal genes, it has been demonstrated that the *ipnA* transcripts start in the corresponding intergenic regions (Fig. 3). The fungal IPNS genes identified thus far do not possess introns (reviewed in reference 49).

Gene Specific for Penicillin Biosynthesis

***aatA* (*penDE*), encoding IAT.** The third and final reaction of penicillin biosynthesis, which does not occur in cephalosporin biosynthesis and has been found in fungi only, is catalyzed by IAT (10, 11, 54, 98, 123, 199, 263, 265, 311). The hydrophilic L- α -AAA side chain is exchanged for a hydrophobic acyl group, e.g., phenylacetyl in penicillin G (Fig. 2). IAT shows a broad substrate specificity (reviewed in references 198 and 211). By addition of appropriate precursor molecules, the fermentation can be directed toward a specific penicillin; e.g., for production of penicillin G, phenylacetic acid is added, whereas for production of penicillin V, phenoxyacetic acid is added. Once the precursor has been taken up, it must be activated to its CoA thioester. This reaction seems to be carried out by phenylacetyl-CoA ligase (53, 171). It is unclear, however, whether a specific enzyme is needed for this reaction, because a possible candidate is the acetyl-CoA synthetase (ACS), which has been purified from *P. chrysogenum* and whose structural gene, *acuA*, has been cloned (215, 216). It was shown that the ACS enzymes of both *P. chrysogenum* and *A. nidulans* have the capability to catalyze in vitro the activation (to their CoA thioesters) of some of the side chain precursors required for the production of several penicillins by these fungi (216).

A two-step enzymatic process for the conversion of IPN to penicillin G has been proposed (265). In the first step, IPN is deacylated to 6-aminopenicillanic acid (6-APA), and in the second step, 6-APA is acylated to penicillin G through the addition of a phenylacetyl group from its CoA derivative (Fig. 2). Thus, two enzymatic functions are required, an amidohydrolase and an acyl-CoA:6-APA acyltransferase function. For many years, it remained unclear whether two enzymes and 6-APA as an intermediate are involved in this step of penicillin biosynthesis. The cloning and sequencing of the gene (Table 3) helped to answer this question. It was found that IAT of *P. chrysogenum* has isopenicillin N amidohydrolase, 6-APA acyltransferase, and penicillin amidase activities, all of which are encoded by the single *aatA* gene (11). 6-APA remains bound to IAT when the enzyme is saturated with appropriate acyl-CoA substrates but is released in their absence. Significant amounts of 6-APA are produced when exogenous side chain precursors are not fed to *P. chrysogenum* (reviewed in reference 264).

The purification of *P. chrysogenum* IAT (9, 10, 199) led to the assumption that the active enzyme is a monomeric protein with a molecular mass of 29 kDa (9, 10). In several partially purified enzyme preparations, however, three proteins of about 40, 29, and 11 kDa were present in ratios that differed among experiments. The discrepancy in the size of IAT among the various purifications was solved by the cloning and sequencing of the corresponding *P. chrysogenum* and *A. nidulans* *aatA* genes independently by Barredo et al. (35) and Tobin et al. (325) (Fig. 3; Table 3). Both groups used purified *P. chry-*

sogenum IAT to determine the N-terminal sequence of the 29-kDa subunit. Based on this information, oligonucleotides were used to identify recombinant bacteriophage lambda EMBL-3 clones which carried a single gene, *aatA*, only. The deduced amino acid sequence showed that the ORF encoded a protein of about 40 kDa. Downstream of this sequence, however, the sequence matched the N-terminal sequence of the 29-kDa protein that was initially purified, implying that the protein was proteolytically processed. The *A. nidulans* gene was also cloned by both groups. By using oligonucleotides derived from the *P. chrysogenum* *aatA* gene, a bacteriophage lambda EMBL-3 clone which was known to contain the *ipnA* gene (325) and a whole bacteriophage lambda EMBL-3 genomic DNA bank of *A. nidulans* were screened (234). Computer analysis revealed that in contrast to the other penicillin biosynthesis genes (*acvA* and *ipnA*), the *aatA* genes contain three introns in both organisms at similar positions (Table 3) (35, 325). This was confirmed by analysis of their respective cDNAs (112, 325). No DNA sequence homologous to the *aatA* gene of *P. chrysogenum* was found in the genomes of three different strains of *A. chrysogenum* (129) and actinomycetes (211). This finding is consistent with the notion that 6-APA: acyltransferase activity which is also carried out by IAT, is lacking in *A. chrysogenum* and other cephalosporin producers (10). Therefore, these organisms do not produce penicillin G or any other penicillins with a hydrophobic side chain.

As mentioned above, the active form of the IAT enzyme was proposed to result from processing of the 40-kDa monomeric precursor to a heterodimer containing subunits of 11 and 29 kDa (10, 35, 325). Further evidence for processing of IAT was provided by Whiteman et al. (341), who purified both the *A. nidulans* and *P. chrysogenum* IAT. When the authors used solutions containing one of the *P. chrysogenum* 11- or 29-kDa proteins only, low levels of activity were measured. This activity could be attributed to an incomplete separation of the two fragments on a gel permeation column. A substantial increase (up to 15-fold) occurred, however, when the solutions containing the subunits were mixed before the assay, indicating that reassociation of the 29- and 11-kDa proteins is required for IAT activity. When both subunits from *P. chrysogenum* were expressed from two different plasmids in *E. coli*, production of either subunit in the absence of the other did not result in active recombinant IAT. However, cotransformation of *E. coli* with two plasmids, each encoding a different IAT subunit, produced recombinant IAT having acyl-CoA:6-APA acyltransferase activity, providing evidence that both subunits are required for activity (323).

To investigate the mechanism of IAT proteolysis in detail, *P. chrysogenum* IAT was overexpressed from the *aatA* gene in *E. coli* and recombinant active IAT was isolated. When purified to homogeneity, recombinant IAT was an α , β -heterodimer, composed of 11-kDa (α) and 29-kDa (β) subunits, derived from a 40-kDa precursor polypeptide by posttranslational cleavage. The processing event that generated the two subunits of recombinant IAT from the 40-kDa precursor polypeptide occurred between Gly 102 and Cys 103 (13). Mutation of *aatA* in the 11-kDa (α) subunit, resulting in replacement of Thr 105 with Asn, led to inactive and uncleaved recombinant IAT. However, coexpression of this mutant *aatA* with an *aatA* derivative encoding the β subunit in *E. coli* produced acyl-CoA: 6-APA acyltransferase activity (323). These results suggest that the formation of recombinant IAT involves cooperative folding events between the subunits. In vitro transcription and translation were used to determine the origin of the IAT hydrolase activity that cleaved the 40-kDa precursor polypeptide. The appearance of a 29-kDa protein (and presumably the corre-

sponding 11-kDa protein, although not observable) from the 40-kDa in vitro-translated protein provided evidence that IAT hydrolysis is an autocatalytic event (323).

Site-directed mutagenesis of the *aatA* gene and expression in *E. coli* revealed that Cys 103 is required for IAT proenzyme cleavage. Whether this requirement reflects a direct participation of Cys 103 in cleavage or as part of a cleavage recognition site has not been clarified yet. However, it cannot be entirely excluded yet that Cys 103 is involved in IAT enzyme activity, because all of these experiments were based on the detection of enzyme specific activity (324). The encoded amino acid sequence in the cleavage site is identical in *P. chrysogenum* and *A. nidulans* (Arg-Asp-Gly...Cys-Thr-Thr) (13, 14, 324).

IAT enzymes seem to be functionally similar to bacterial penicillin and cephalosporin acylases that catalyze the deacylation of acyl side chains of penicillins and cephalosporins to yield 6-APA and 7-aminocephalosporanic acid, respectively. The penicillin acylase from *E. coli* ATCC 11105 is a periplasmic enzyme which consists of two nonidentical subunits (40) that are produced by posttranslational processing from a precursor protein (41). This functional similarity between fungal IATs and bacterial acylases is striking. However, there is only very low sequence similarity (approximately 11% identical amino acids) between fungal IATs and the *E. coli* acylase.

Genes Specific for Cephalosporin Biosynthesis

***cefD*, encoding IPN epimerase.** The reaction catalyzed by IPN epimerase directs the pathway (Fig. 2) to the production of cephalosporins. IPN epimerase catalyzes the epimerization of the L- α -AAA side chain of IPN to the D enantiomer to give penicillin N, which is the precursor of antibiotics containing the ceph-3-em nucleus, i.e., cephalosporins and cephamycins (7-methoxycephalosporins) (151, 174) (Fig. 2). The purification of *A. chrysogenum* IPN epimerase proved to be difficult because the enzyme was extremely labile in cell-free preparations (174). So far, no further data on the fungal protein or gene are available. Therefore, it is still unknown whether the putative *cefD* gene is part of one of the *A. chrysogenum* clusters (Fig. 3).

The *S. clavuligerus* IPN epimerase was found to be more stable and was characterized biochemically (155). The corresponding gene (*cefD*) was cloned and sequenced. *cefD* is located immediately upstream of the gene encoding the DAOC synthetase (expandase) (*cefE*) (176) (Fig. 3). *cefD* encodes 398 amino acid residues with a deduced molecular mass of 43,497 Da. The cloning of the *cefD* genes of *Streptomyces lipmanii* (61, 299), *Nocardia lactamdurans* (77), and *Lysobacter lactamgenus* YK90 (166, 167) has been reported (Fig. 3).

P. chrysogenum expresses no IPN epimerase activity, and a probe of the *S. lipmanii* *cefD* gene did not hybridize to DNA of *P. chrysogenum* (61). However, some minor IPN epimerase activity exists in *P. chrysogenum* (12), because recombinant strains of *P. chrysogenum* expressing the *S. clavuligerus* *cefE* gene encoding DAOC synthetase were found to produce DAOC (12, 82) (Fig. 2). Since the DAOC synthetase shows no affinity toward IPN, isomerization of IPN into penicillin N must have occurred (94, 182). However, Alvi et al. (12) emphasized that IPN epimerase activity in *P. chrysogenum* could be due to some unspecific amino acid racemase. Hence, these experiments do not necessarily imply the presence of a *cefD*-like gene in *P. chrysogenum*.

***cefEF*, encoding DAOC synthetase (ring expandase)/DAC hydroxylase.** In cephalosporin biosynthesis, penicillin N is converted to DAOC by expansion of the five-membered thiazolidine ring to give the six-membered dihydrothiazine ring of

DAOC (Fig. 2). This reaction is catalyzed by DAOC synthetase, which possesses the required expandase function (172). The enzyme was purified from *A. chrysogenum* (93, 182), *S. clavuligerus* (155), and *S. lactamdurans* (81). Fungal and bacterial expandases are stimulated by reducing agents, like dithiothreitol or glutathione, and show an absolute requirement for Fe²⁺, molecular oxygen, α -ketoglutarate, ascorbate, and possibly ATP. These unusual cofactor requirements are characteristic of the class of intermolecular dioxygenases which activate oxygen in the decomposition of equimolar amounts of α -ketoglutarate to form carbon dioxide and succinate (182; reviewed in reference 245).

In the following reaction step, the methyl group at C-3 of DAOC is hydroxylated and oxidized to give DAC (93, 290). This reaction is catalyzed by DAC hydroxylase, which is very similar to DAOC synthetase. The enzyme also belongs to the group of α -ketoglutarate-linked dioxygenases.

Ring expansion by DAOC synthetase and the hydroxylation reaction are both carried out by the same peptide in *A. chrysogenum* (93, 284, 290). Purification of this enzyme, determination of its N-terminal amino acid sequence, and reverse genetics allowed the cloning of the structural gene, designated *cefEF* (284). Expression of the cloned *cefEF* gene in *E. coli* (284) established unequivocally that in *A. chrysogenum* one protein is responsible for the ring expansion of penicillin N to DAOC and the 3' hydroxylation of DAOC to DAC (Fig. 2; Table 4). In contrast, in *S. clavuligerus*, the two enzymatic activities were separated by anion-exchange chromatography (156) and were later found to be encoded by two genes, *cefE* and *cefF* (Table 4; Fig. 2).

The *cefEF* gene of *A. chrysogenum* is located on chromosome II (300). It is closely linked to the *cefG* gene but is transcribed in the opposite direction (Fig. 3). The intergenic region of about 1 kb is believed to contain the promoters for both genes (131). In *S. clavuligerus*, the *cefF* gene is closely linked to the *cefD* and *cefE* genes but is transcribed in the opposite orientation (Fig. 3) (175). In *Lysobacter lactamgenus* and *Nocardia lactamdurans*, different orders of genes were found (75, 77, 79, 166, 167) (Fig. 3).

The only data on regulation of the *A. chrysogenum* gene thus far reported is a Northern blot analysis which showed that a 1.2-kb transcript corresponding to the *cefEF* gene was detectable in cells after 48 h of growth in a defined production medium (131).

Gene Specific for Cephalosporin C Biosynthesis in Fungi

***cefG*, encoding acetyl-CoA:DAC acetyltransferase.** In the last step of the cephalosporin C biosynthesis, which is best studied in the fungus *A. chrysogenum*, an acetyl moiety from acetyl-CoA is transferred to the -OH group of DAC; this step is catalyzed by acetyl-CoA:DAC acetyltransferase (108, 122) (Fig. 2). The corresponding structural gene (*cefG*) of *A. chrysogenum* was cloned and sequenced independently by three groups (Table 5). The *cefG* gene was cloned based on its close linkage to *cefEF*. Sequencing of the region adjacent to *cefEF* led to the identification of an ORF, and a DNA fragment encoding this ORF allowed complementation of the *A. chrysogenum* mutant M40, which is deficient in acetyl-CoA:DAC acetyltransferase activity. Thus, the identified ORF most probably contained the *cefG* gene, which was confirmed by overexpression of the gene in *A. niger*, which naturally lacks such an activity (219). Gutiérrez et al. (131) screened an *A. chrysogenum* bacteriophage lambda library with a probe specific for the *cefEF* gene. Northern blotting and DNA sequence analysis revealed the existence of the *cefG* gene close to the *cefEF* gene.

The authenticity of the *cefG* gene was proved by complementation of *A. chrysogenum* ATCC 20371, which lacks acetyl-CoA:DAC acetyltransferase activity. Matsuda et al. (220) cloned the gene by screening a cDNA library with oligonucleotides based on the N-terminal sequence of the enzyme. In addition, they proved the cloning of *cefG* by performing a gene disruption (replacement) experiment. The *cefG*-disrupted strains lacked the ability to produce cephalosporin C and accumulated its precursor, DAC, in the culture broth (221).

cefG contains two introns, as demonstrated by sequencing of its cDNA (219, 220). It is closely linked to *cefEF* but is transcribed in the opposite orientation. The size of the separating intergenic region is not clear, because Gutiérrez et al. (131) found 938 bp, Mathison et al. (219) found 1,077 bp, and Matsuda et al. (220) reported a 1,114-bp intergenic region (Fig. 3; Table 5).

Northern blot analysis of *A. chrysogenum* RNA showed a very weak transcript of about 1.4 kb, corresponding to the *cefG* gene, in cells grown in a defined production medium for 48 and 96 h (131, 219). These findings seem to agree with reports on the late conversion of DAC to cephalosporin C in cephalosporin fermentations (344). *cefEF* appears to be expressed at an earlier stage of the fermentation, suggesting that *cefEF* and *cefG* are expressed differently from their intergenic region (131) (Fig. 3).

COMPARTMENTALIZATION OF GENE PRODUCTS

The penicillin biosynthesis pathway occurs in different compartments of the cell (Fig. 4). For localization of the ACVS protein, subcellular fractions obtained from protoplasts of a high-penicillin-producing *P. chrysogenum* strain (BC 1505) were analyzed. Because ACVS protein was detected in both the membrane and the soluble fraction of purified vacuoles, it was concluded that it is located either within or bound to the vacuolar membrane (187). In addition, a large portion of cellular L- α -AAA, which is most probably used for β -lactam synthesis, is also contained in the vacuoles and thus is sequestered from the cytosol (5, 143) (Fig. 4).

P. chrysogenum IPNS protein was found in the cytoplasm, whereas IAT was detected in organelles with a diameter of 200 to 800 nm, which were assumed to be microbodies (238) (Fig. 4). The latter result has been supported by the finding that the *P. chrysogenum* IAT contains a putative targeting signal sequence, a C-terminal alanine-arginine-leucine (34, 125). The importance of this sequence was proved by deleting it in vitro. After transformation of the *P. chrysogenum* *npe6* strain lacking IAT specific activity, the mutated enzyme was located in vacuoles and the neighboring cytoplasm. Although IAT was produced in vivo, as shown by Western blot analysis and by measurement of IAT specific activity in vitro, the mutants did not produce penicillin (237), indicating that targeting of the enzyme to microbodies is essential for penicillin biosynthesis. Furthermore, a positive correlation between the capacity for penicillin production and the number of organelles per cell was observed when different *P. chrysogenum* strains were compared (238). Hence, the biogenesis of organelles and the genes responsible for this process might have an impact on the penicillin production. The localization of the penicillin biosynthesis in three different cellular compartments reflects the complexity of this biosynthetic pathway. Several transport steps are thus required to bring intermediates of the penicillin biosynthesis pathway together with the enzymes.

Although the presence in *P. chrysogenum* Wis54-1255 of a transport system for the side chain precursor phenylacetic acid was reported (113), subsequent investigations showed that

phenylacetic acid passes the plasma membrane via passive diffusion of the protonated species (140).

ORIGIN OF β -LACTAM BIOSYNTHESIS GENES IN FUNGI

β -Lactam biosynthesis genes have been found both in bacterial species and in fungi. The availability of sequence information about bacterial and fungal genes led to speculations about their evolutionary relationship. Based on several observations, a horizontal transfer of β -lactam biosynthesis genes from bacteria to fungi during evolution has been proposed by several authors (63, 184, 254, 339). This hypothesis was recently questioned by Smith et al. (308). The arguments in favor of a horizontal gene transfer are as follows. (i) *ipnA* genes of fungi and bacteria show high sequence similarities. More than 60% of the nucleotide bases and 50% of the deduced amino acids are identical. (ii) Bacterial as well as fungal β -lactam genes are organized in clusters. The β -lactam biosynthesis genes in bacteria are organized into a single cluster, as are the penicillin biosynthesis genes in fungi (Fig. 3). The cephalosporin biosynthesis genes in *A. chrysogenum* are organized into two clusters located on different chromosomes (Fig. 3). This finding led to the assumption that the β -lactam biosynthesis genes were transferred as a single cluster from an ancestral prokaryote to a common ancestor of the β -lactam-synthesizing fungi. In the eukaryotic ancestor, the biosynthesis genes were split between two chromosomes. One part encodes the early genes of β -lactam biosynthesis, and the other encodes the late genes. Later in the lineage, an ancestor of *A. nidulans* and *P. chrysogenum* diverged from *A. chrysogenum* and has presumably lost the second cluster with the genes for the late stage of cephalosporin biosynthesis (299) (Fig. 3). (iii) The G+C content in the third position of codons containing the *ipnA* gene of *A. nidulans* and *P. chrysogenum* is unusually high and could indicate an evolutionary origin from streptomycetes, which show G+C contents of greater than 70% (8). (iv) Fungal *acvA* and *ipnA* genes do not contain introns, indicating a bacterial origin of the genes.

In addition, Aharonowitz et al. (8) proposed that during the evolution of β -lactam biosynthesis genes, *Streptomyces* spp. must have evolved specific resistance mechanisms to avoid self-toxicification. If the transfer had occurred from fungi to bacteria, it would have been lethal for bacteria. Hence, the transfer is more likely to have occurred from bacteria to fungi, which would not have been forced to evolve resistance mechanisms because of their lack of susceptibility. In contrast to the other penicillin biosynthesis genes, the *aacA* genes contain introns. On the basis of linkage of the *aacA* and *ipnA* genes (Fig. 3), Skatrud (299) suggested that a sequence functionally related to *aacA* was transferred together with the β -lactam genes and was later modified to its current functional form. Since IAT possesses amidohydrolase activity to deacylate IPN to 6-APA (11) (Fig. 2), which shows a weak antibiotic activity only, an ancestral amidohydrolase activity in the prokaryotic ancestor might have had a resistance function. Its corresponding gene might have been fused in fungi with a eukaryotic gene (299). Genetic linkage of antibiotic synthesis genes and resistance genes is common in prokaryotes (294). Based on the DNA sequences of *ipnA* genes from gram-positive streptomycetes and from fungi and a rate of nucleotide substitution of 10^{-9} nucleotide change per site per year (188), Weigel et al. (339) proposed that the transfer occurred 370 million years ago. The cloning and sequencing of an *ipnA* gene from a gram-negative bacterium, *Flavobacterium* sp., however, led to an extension and modification of the hypothesis of horizontal

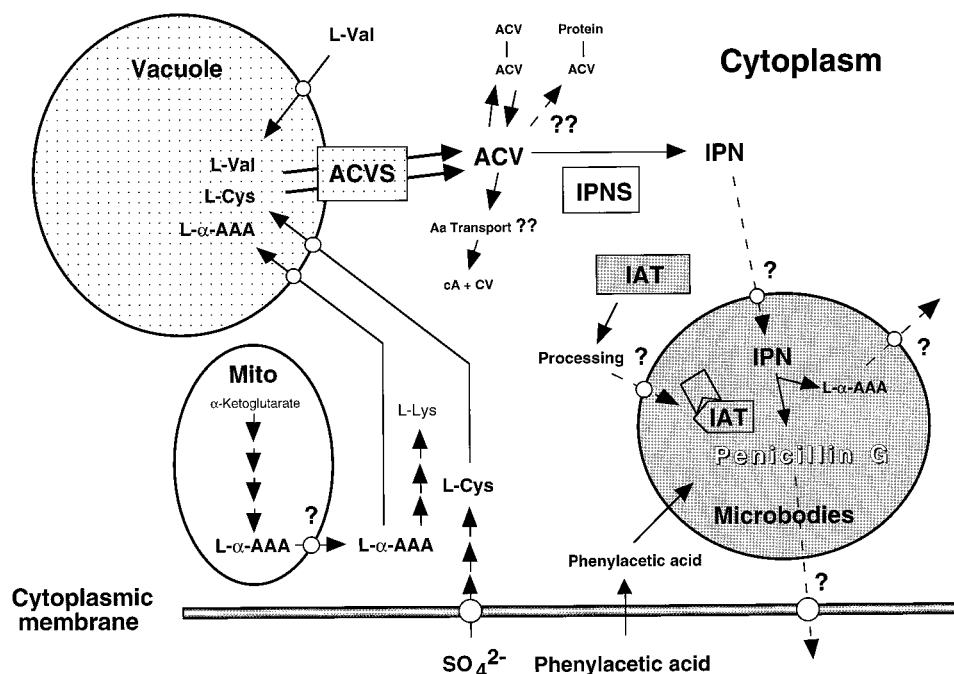


FIG. 4. Compartmentalization of penicillin biosynthesis gene products. Enzyme names are boxed. Reactions which are hypothetical are labelled by two question marks. Most of the transport processes (indicated by a single question mark) which seem likely to exist because of the compartmentalization of the different enzymes have not been elucidated yet. The ACVS seems to be located within or bound to the vacuolar membrane. IPNS and IAT are located in the cytoplasm and in microbodies, respectively. See the text for details. The stage at which the processing of IAT occurs remains to be determined. Abbreviations: Aa, amino acids; cA, cyclized L- α -AAA (6-oxo-piperidine-2-carboxylic acid [Fig. 6]); CV, L-cysteinyl-D-valine; Mito, mitochondrion.

gene transfer. The *ipnA* gene of *Flavobacterium* sp. is 69% identical to the streptomycete gene and 64 to 65% identical to the fungal genes (*A. chrysogenum* and *P. chrysogenum*) (73). A recent reevaluation of the divergence times of organisms by using a protein clock suggested that gram-positive and gram-negative bacteria split about 2 billion years ago and that prokaryotes and a eukaryotic ancestor split about 3.2 to 3.8 billion years ago (111). If the gene transfer from streptomycetes to fungi had occurred only 370 million years ago, as proposed by Weigel et al. (339), it could be expected that the fungal and streptomycete genes would show a greater similarity than the gram-positive (streptomycete) and gram-negative (*Flavobacterium* sp.) bacterial genes. As outlined above, this is not the case (73). Hence, Aharonowitz et al. (8) suggested that multiple gene transfer events might have occurred from bacteria to fungi. It is difficult to imagine, however, why these multiple gene transfers then happened at about the same time as would be expected from the degree of similarity among the proteins of the various organisms. In addition, Smith et al. (308) argued against a horizontal transfer. The authors pointed out that the hypothesis of a horizontal gene transfer, e.g., of the *ipnA* gene, was made on the basis of a very limited data set and was based solely on assumptions about rates of change. They compared the similarity of both IPNS of *A. nidulans*, *P. chrysogenum*, *A. chrysogenum*, *S. clavuligerus*, *S. anulatus*, and *Flavobacterium* sp. and DAOC synthetase of *S. clavuligerus* and *A. chrysogenum*. Based on these similarities, they constructed a tree with conventional evolutionary descent. The authors argued that the simplest interpretation is that the genes for the two enzymes resulted from a duplication that occurred before the prokaryote-eukaryote divergence. The topology of the tree rooted with the duplicated enzymes, the depth of the bacterial branches, and the different orientations of the genes in fungi and eubacteria all appear to be consistent with an ordinary

evolution for IPNS. However, if the genes appeared very early in the evolution, why have most of the eukaryotes and fungi lost the gene cluster? This question cannot be satisfactorily answered at the moment. Thus, the evolutionary origin of β -lactam biosynthesis genes remains speculative.

REGULATORY CIRCUITS AND REGULATORY GENES

Expression of Biosynthesis Genes under Standard Fermentation Conditions

Studies of the expression of penicillin biosynthesis genes were performed mainly with the *E. coli* reporter genes *lacZ* and *uidA*, encoding β -galactosidase (β -Gal) and β -glucuronidase (β -Glu), respectively (see, e.g., references 46, 124, and 194). Most of the results based on the analysis of gene fusions were supported by Northern or Western blot analysis or determination of enzyme specific activities and penicillin titers.

These studies led to the finding that the promoter strengths of penicillin biosynthesis genes are rather different. It was shown that in *A. nidulans*, *aatA* had lower expression than *ipnA* and threefold-higher expression than *acvA* (46, 194). A similar observation was made for the corresponding *acvA* and *ipnA* genes of both *P. chrysogenum* and *A. chrysogenum*. On the basis of reporter gene fusions, it became evident that in both fungi the expression of *acvA* was much weaker than that of *ipnA* (109, 230). The intergenic regions between *acvA* and *ipnA* thus seem to contain the information required for the remarkable difference in expression levels between *acvA* and *ipnA*. The low expression of *acvA* is, at least in wild-type strains of *A. nidulans*, rate limiting for penicillin production, because overexpression of *acvA* led to drastically increased production of penicillin (165) while similar overexpression of *ipnA* and *aatA* did not (112).

It seems reasonable to assume that the expression of penicillin biosynthesis genes is coordinated to ensure the synthesis of penicillin by the concomitant appearance of all gene products. But how is coordination achieved? Biosynthesis genes could be expressed simultaneously; i.e., the genes could be activated by the same regulatory factors. Alternatively, the expression of biosynthesis genes could be sequentially induced.

Ramos et al. (268) showed that a mutant of *A. chrysogenum* (N-2), incapable of producing the β -lactam cephalosporin, lacked IPNS, IPN epimerase, and DAOC synthetase (expandase) activities (Fig. 2). Subsequent investigations revealed that strain N-2 encodes an inactive IPNS caused by a single C-to-T mutation within the coding region of the *ipnA* gene. It was postulated that a functional IPNS or its biosynthetic product IPN might be necessary for the regulation of the later stages of the biosynthesis, i.e., induction of the *cefD* and *cefEF* expression, respectively (Fig. 2 and 3) (269). Furthermore, Hoskins et al. (145) disrupted the *acvA* gene of *A. chrysogenum*. Although the predicted alterations of the target gene were not detected, the authors demonstrated IPNS activity in non-cephalosporin-producing transformants. This suggested that the *ipnA* gene can be expressed without the presence of precursor tripeptide molecules. Hence, in *A. chrysogenum*, the *ipnA* gene seems to be coordinately regulated whereas the later genes of the cephalosporin pathway (*cefD* and *cefEF*) (Fig. 3) appear to be sequentially induced.

To further study these observations, *acvA* was disrupted in a strain of *A. nidulans* (47, 305). This strain had a disrupted *acvA* gene on chromosome VI and, in addition, reporter gene fusions of the penicillin biosynthesis genes integrated in single copy at the chromosomal *argB* gene locus on chromosome III. *acvA*, *ipnA*, and *aataA* gene fusions were expressed at the same level in this strain as in the nondisrupted strain (47, 194). This was confirmed by determining IPNS and IAT specific activities and by Western blot analysis of IPNS, which showed the presence of both enzymes in an *acvA*-disrupted strain (47, 194). Hence, the genes were expressed despite the lack of precursor ACV and IPN molecules, indicating that in contrast to the cephalosporin biosynthesis genes, none of the penicillin biosynthesis genes are sequentially induced and their expression is most probably coordinated. This is also supported by the similar time course in the expression of the *A. nidulans acvA* and *ipnA* genes (46) and by the appearance of transcripts of all three penicillin biosynthesis genes in *P. chrysogenum* at the same time during a fermentation run (273).

However, some observations make the view of a coordinated expression of the genes more complicated. Differential regulation of *acvA*, *ipnA*, and *aataA* in *A. nidulans* has been observed in response to exogenous signals. While the expression of *A. nidulans acvA* and *ipnA* genes was significantly repressed by L-lysine, *aataA* expression was not (48, 191). Furthermore, in fermentation medium, only *ipnA* was significantly repressed by glucose (46, 194).

It is interesting that in fermentation medium, *acvA* and *ipnA* gene fusions were expressed for up to 68 and 46 h, respectively (46). In contrast, *aataA* expression was detected for only about 24 h. In this respect, the temporal expression pattern of *aataA* is more similar to that of an *oliC-lacZ* gene fusion; *oliC* encodes subunit 9 of the *A. nidulans* mitochondrial ATP synthase complex and is regarded as a gene of the primary metabolism (46, 194).

In contrast to the other penicillin biosynthesis genes, the *aataA* genes of both *A. nidulans* and *P. chrysogenum* encode three introns (Table 3; Fig. 3). In theory, introns could play a role in regulating *aataA* expression. This was also suggested by the observation that when the inducible *alcA* promoter was

used for the expression of *aataA*, the use of *aataA* cDNA gave higher IAT specific activity than did the use of the genomic gene carrying all three introns (112). However, a regulatory role of the first intron in the wild-type gene is unlikely, since there was no difference in the expression pattern of an *A. nidulans aataA-lacZ* fusion compared with an *aataA-lacZ* fusion, which had the first intron included (194). It is conceivable, however, that the second and/or third intron plays a regulatory role or, alternatively, that excision of introns becomes limiting in strains in which the steady-state level of *aataA* mRNA is strongly increased (112).

Previous studies of the formation of secondary metabolites in batch fermentations led to the definition of two phases: the growth phase (trophophase) and the period of secondary-metabolite production (idiophase) (reviewed in reference 210). A strict separation of the two phases, with production of secondary metabolites being virtually restricted to the idiophase, has been observed in antibiotic-producing bacterial cultures. It was first reported that the expression of the *ipnA* gene encoding IPNS in *A. nidulans* is temporally delayed in a similar manner to that of expression of bacterial secondary-metabolism genes (124). However, when lactose was used as the sole carbon source, there was no sharp separation of the trophophase and idiophase with respect to penicillin production or the expression of penicillin biosynthesis genes (46, 194). The previously observed delayed expression of *ipnA* is due to the use of glucose as the carbon source (101), which repressed transcription and expression of the *ipnA* gene (46, 101) (Fig. 5). For some secondary metabolites, it seems likely that their production in a clear-cut idiophase reflects the inhibiting effects of certain compounds in the medium (46, 101) rather than an intrinsic temporal delay in the product formation pattern. This view is consistent with the results of Renno et al. (273), who showed that in wild-type *P. chrysogenum* (NRRL 1951) and the high-penicillin producing strain P2, the highest steady-state level of mRNAs of all penicillin biosynthesis genes was observed during maximal growth in both shake flasks and 2-liter fermentors.

However, some observations imply that in *P. chrysogenum* there might be a temporal expression of β -lactam biosynthesis genes. *ipnA* steady-state mRNA levels increased with the age of the culture, indicating preferential transcription of the gene at late growth times (316). This is consistent with the observation of Feng et al. (109) that expression of an *ipnA-uidA* gene fusion was detectable after only 24 h in fermentation medium with lactose as the carbon source whereas an *acvA-uidA* gene fusion seemed to be expressed from the beginning of a fermentation run. Hence, there might even be differences in the temporal expression of genes of the same cluster as well as in different fungi.

Promoter Structures of the *A. nidulans* Genes *acvA* and *ipnA*

So far, only two studies have analyzed the promoters of β -lactam biosynthesis genes in the homologous host in detail. Pérez-Esteban et al. (257) reported a comprehensive deletion analysis of the *A. nidulans ipnA* promoter in which the promoter region was fused in frame with the *E. coli lacZ* reporter gene (encoding β -Gal). Integration of such deletion constructs in single copy at the chromosomal *argB* gene locus and determination of the β -Gal specific activity of the different *A. nidulans* transformant strains revealed that several regions of the promoter were involved in the basal expression of *ipnA* and the repression by sucrose (see "Carbon source regulation" below).

In this deletion study, the expression of the divergently transcribed *acvA* gene (Fig. 3) was not determined. Divergently

transcribed genes, however, provide an interesting case of regulation, because possible functional interactions between the promoters could exist and single *cis*-acting sites might have the potential to regulate two genes simultaneously. Hence, a moving-window analysis was carried out; i.e., nested deletions were introduced into the intergenic region between *A. nidulans* *acvA* and *ipnA*. The ATG start codons of both genes were fused in frame with the two *E. coli* reporter genes *lacZ* and *uidA* (encoding β -Gal and β -Glu), respectively, and these deletion constructs were integrated in single copy at the chromosomal *argB* gene locus. Hence, the effects of deletions on the expression of both genes were measured simultaneously within one transformant strain by determining β -Gal and β -Glu specific activities (319). These deletion analyses revealed that the intergenic region between *acvA* and *ipnA* contains several regions with *cis*-acting DNA elements. Thus far, no element was found in the intergenic region which affected the expression of one gene only, although differential expression of genes, e.g., as a result of the addition of amino acids to the medium, was observed. Taken together, these findings suggest that the promoters of both genes are, at least in part, physically overlapping and share common *cis*-acting elements (319).

Carbon Source Regulation

Industrial production of penicillin by *P. chrysogenum* was previously carried out with lactose as the C source, since it gave the highest penicillin titer. The use of excess glucose leads to a drastic reduction of the penicillin titer (309). Fructose, galactose, and sucrose also have a negative effect on penicillin production, but lactose does not (276). Nowadays, this problem has been partially overcome by feeding subrepressing doses of glucose and by using lactose as the C source (317). Since in general the fungus grows better with glucose than with lactose (46), the production of penicillin appears to be favored by suboptimal growth conditions.

C-source regulation seems to act at several points of the penicillin biosynthesis: (i) flux of L- α -AAA, (ii) activation of side chain precursors, (iii) transcription of penicillin biosynthesis genes (Fig. 5), and (iv) posttranscriptional regulation of penicillin biosynthesis genes.

The replacement of lactose by glucose reduced the L- α -AAA pool in mycelia of *P. chrysogenum* and thus probably the flux of L- α -AAA to ACV (144 (see "Amino acids as precursors and mediators of regulation" below). The formation of ACV and IPN was also repressed by glucose (277) (Fig. 2). Indirect measurements based on the incorporation of [14 C]valine into ACV in vivo suggested that the glucose repression of penicillin biosynthesis involves repression but not inhibition of penicillin-synthesizing enzymes. Furthermore, the specific activity of IPNS but not of IAT was reduced in cells of a penicillin-producing strain (AS-P-78) grown on glucose. These results suggested that glucose represses the formation of ACVS and IPNS in *P. chrysogenum* (144, 276, 277). In agreement with these findings was the observation that the expression of both the *acvA* and *ipnA* genes of *P. chrysogenum* Q176, both measured by using the *uidA* reporter gene, was repressed by glucose (109) (Fig. 5). Renno et al. (273) claimed, however, that steady-state mRNA levels of all three *P. chrysogenum* penicillin biosynthesis genes were highest during rapid growth, when considerable levels of glucose were present. This shows that measurement of carbon regulation depends, at least in part, on the experimental approach used.

In *A. nidulans*, a similar phenomenon was observed. The use of repressing carbon sources such as glucose or sucrose in the fermentation medium reduced the amount of penicillin pro-

duced (46, 101). Results obtained with reporter gene fusions showed that the expression of the *ipnA* gene was repressed when glucose or sucrose was used instead of lactose as the C source during fermentation (46, 101) (Fig. 5). This was further supported by the finding that the IPNS specific activity was drastically reduced in glucose-grown mycelia (46). Repression of *ipnA* expression by repressing C sources occurs, at least in part, at the transcriptional level, because the steady-state level of *ipnA* mRNA decreased when mycelia were cultivated with repressing C sources such as sucrose (101).

Unexpectedly, the expression of both *acvA* and *aatA* reporter gene fusions was repressed only slightly, if at all, by glucose in fermentation medium (46, 194). However, the specific activity of the *aatA* gene product, IAT, was reduced in mycelia grown with glucose instead of lactose (46, 194). This suggests that the glucose regulation of IAT takes place, at least in part, posttranscriptionally. The effect of glucose on IAT specific activity could be reversed by washing cells and reincubating them in lactose-containing medium (194). It is unclear why glucose regulation occurs, in the case of *ipnA*, transcriptionally or, in the case of *aatA*, apparently posttranscriptionally.

The results obtained with *A. chrysogenum* seem to be similar to those obtained with *A. nidulans*. Cephalosporin C formation by *A. chrysogenum* depends on the C source used (85). C sources which support the most rapid growth, like glucose or glycerol, had a negative effect on β -lactam production (87, 223). Fermentations with different C sources showed that glucose exerted a much greater negative effect on cephalosporin C than on penicillin N formation, suggesting that it had a more pronounced effect on the later steps of the pathway (36). The ACVS level was not affected by higher concentrations of glucose, making a major repressive effect of glucose at the level of gene expression unlikely (349). However, ACVS specific activity measured in crude protein extracts was severely inhibited by glucose and glycerol. The inhibition could be reversed by the addition of ATP. Interestingly, the purified enzyme was not inhibited by glucose but was inhibited by glyceraldehyde-3-phosphate and glyceraldehyde (346). From these observations, the authors concluded that inhibition of ACVS specific activity in crude extracts, by glucose and some other sugars, is due to depletion of the cofactor ATP via sugar metabolism.

In contrast to the penicillin production strain *P. chrysogenum* AS-P-78, investigated by Revilla et al. (277), the IAT specific activity of both *A. nidulans* and the *P. chrysogenum* wild-type strain NRRL 1951 was clearly reduced in glucose-grown cultures (46). Glucose was also found to cause inactivation of *P. chrysogenum* acetyl-CoA synthetase, which can catalyze the activation (to their CoA thioesters) of some of the side chain precursors required for the production of several penicillins in vitro (216). The contributions to the overall reduction of the penicillin titer by each of the steps affected by glucose are not yet clear.

Previously, it was reported that the uptake of side chain precursors of phenylacetic acid was regulated by glucose (see, e.g., reference 113). However, it was recently shown that phenylacetic acid passes the plasma membrane via passive diffusion of the protonated species (140), thus excluding the possibility that the uptake could be regulated by the available C source.

An involvement of cyclic AMP (cAMP) in C-source regulation is controversial. In *P. chrysogenum*, cAMP levels were high during growth on lactose and decreased markedly (by about 75%) when glucose was added (178). In *A. nidulans*, however, a correlation between intracellular cAMP levels and the C source (lactose and glucose) was not detected (44).

To study the molecular basis of C-source regulation of *ipnA*

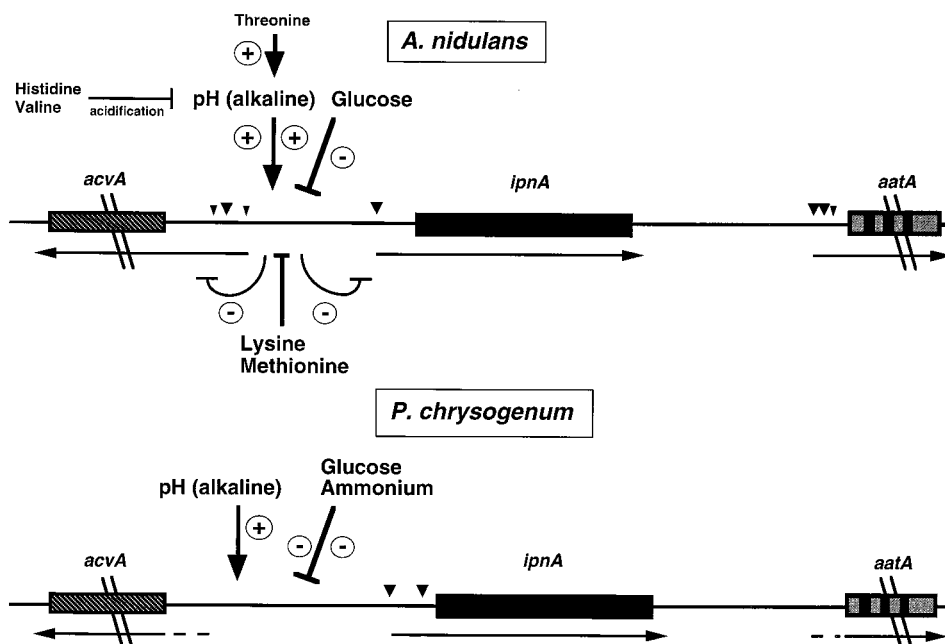


FIG. 5. Regulatory circuits affecting the expression of the penicillin biosynthesis genes *acvA* and *ipnA* in *A. nidulans* and *P. chrysogenum*. Large and small arrows indicate major and minor transcription start sites, respectively. Transcript start sites of *acvA* and *aatA* in *P. chrysogenum* have not been reported yet. Until now, in *P. chrysogenum* the effect of alkaline pH has been shown for the *ipnA* gene (316). The addition of certain amino acids to the medium results in the indicated effects on gene expression. Some of these effects are mediated by the ambient pH. See the text for details. Regulatory circuits affecting the expression of the *aatA* gene in both fungi have not been identified yet.

expression, several mutants of *A. nidulans* carrying previously characterized loci affecting the glucose repression of several genes of the primary metabolism (*creA*^{d-1}, *creB*₃₀₄, and *creC*₃₀₂) (21, 146) have been analyzed. In these mutants, penicillin production was still reduced by glucose (46, 49). However, Espeso and Peñalva (101) observed in mutants with extreme loss-of-function mutations in *creA* slightly derepressed *ipnA* steady-state transcript levels. This was consistent with a deletion analysis of the *ipnA* promoter, demonstrating that a *cis*-acting DNA region crucial to sucrose repression maps between -1334 and -966 relative to the transcriptional start site of the *ipnA* gene (257). A single CREA binding site was detected in this region, which was protected in DNase I footprint analysis by using a glutathione *S*-transferase (GST)-CREA protein which contained amino acids 35 to 240 of CREA (104). The full-length CREA polypeptide deduced from the gene consists of 415 amino acids (95). To test the functionality of this putative CREA binding site in vivo, the effect of its deletion was measured in an *ipnA-lacZ* gene fusion. The expression of the gene fusion with a deleted CREA binding site was still repressed when sucrose instead of lactose was used as the C source (104). Hence, it is unlikely that CREA plays a role in C-source repression of penicillin biosynthesis. This also agrees with the finding that acetate and glycerol, which are repressing and derepressing C sources, respectively, of some primary-metabolism genes in the *creA*-mediated circuit of carbon catabolite repression, behaved in the opposite manner to what would be expected from *creA* control. The use of acetate led to increased steady-state levels of *ipnA* transcript and penicillin titers, and the use of glycerol led to the opposite effects, i.e., decreased *ipnA* transcript levels and penicillin titers (104).

Previously, it was shown that in *creB* and *creC* mutants the production of penicillin was still reduced when glucose instead of lactose was used as the C source (46, 49). Additional experiments further excluded the possibility of a direct involvement

of *creB* and *creC* mutations in C-source repression of *ipnA* transcription (100). Thus, the mechanism(s) of regulation of penicillin biosynthesis by repressing C sources remains to be elucidated.

An interesting observation was made by Kennedy and Turner (165). In *A. nidulans* strains such as WG355 or R21, glucose usually led to a reduction of the penicillin titer to about 30 to 50% (46, 101), whereas with strain G191, a reduction to about 10% was measured. This might represent an unexpected strain difference, with penicillin production in G191 being more sensitive to glucose repression (165). Strain G191, for example, carries a mutation in the *uaY* gene (*uaY*⁹), which encodes a transcriptional activator for a number of unlinked genes involved in purine utilization (315), or cryptic mutations could be present in G191 which could be responsible for this unexpected observation. It seems worth investigating the difference between strain G191 and other strains with respect to carbon regulation of penicillin biosynthesis.

pH Regulation Mediated by the Transcriptional Factor PACC

Penicillin production is subject to regulation by ambient pH (104, 295) (Fig. 5). Wild-type strains of *A. nidulans* can grow in media over the pH range of 2.5 to 10.5 (282). There was markedly more penicillin in the culture broth when the pH of the medium was kept constant at 8.1 than when it was kept constant at 6.5 or 5.1 (295). Control experiments excluded the possibility that the difference in penicillin yield measured was due to the higher stability of penicillin G at alkaline pH (104).

To study the molecular basis of this interesting phenomenon, mutations involved in the pH regulatory network of *A. nidulans* were isolated and analyzed (19, 92, 104, 295). Previously, Dorn (92) had identified mutations specifically affecting the pH regulation of the formation of alkaline and acidic

phosphatases. These mutations were divided into two distinct groups (*pal* and *pac*) based on the phenotypes they encoded. Mutations in some of the *pal* genes (*palA*, *palB*, *palC*, *palE*, and *palF*), which are phenotypically indistinguishable, have been shown to mimic the effects of growth at acidic pH (57). Arst et al. (19) identified two new *pal* genes, *palH* and *palI*, and demonstrated that *palB* and *palE* actually represented the same gene. Thus far, there are a total of six discrete regulatory *pal* genes (*palA*, *palB*, *palC*, *palF*, *palH*, and *palI*) (19). The alkaline phosphatase and acidic phosphatase structural gene were designated *palD* and *pacA*, respectively (reviewed in reference 18). The regulatory *pal* mutations impair the pH regulation of the syntheses of extracellular enzymes, such as phosphatases and permeases. Extracellular enzymes and permeases are not protected by the intracellular pH homeostatic system; therefore, their synthesis is controlled by the external pH. This mode of regulation avoids the synthesis of, for example, secreted alkaline phosphatase in an acidic environment (57).

Mutations at the *pacC* locus result in the phenotypic mimicry of growth under alkaline conditions (57). It has also been noted that *pacC* mutations themselves can be divided into two classes defined by the phenotype when cultured at neutral pH: those that mimic growth under alkaline conditions (the type originally observed) and those (including a null allele) that mimic growth under acidic conditions (57, 322).

PacC mutants mimicking the effect of growth at alkaline pH produced about twice as much penicillin as did wild-type strains, whereas strains carrying *pal* mutations mimicking growth at acidic pH showed the lowest penicillin titers. These findings are consistent with the observation that penicillin titers were higher when wild-type strains were grown at alkaline pH in wild-type strains (295).

The *pacC* gene was cloned and sequenced by complementation of an *argB2 pacC¹⁴* (mimicking alkaline growth conditions) recipient by using a genomic *A. nidulans* library constructed in an *argB⁺*-containing plasmid. The DNA sequence of the *pacC* gene consists of 2,172 bp interrupted by two introns of 85 and 53 bp. The 678-residue-derived protein (M_r 72,939) revealed that PACC contains three putative Cys₂His₂ zinc fingers (322). At alkaline ambient pH, PACC activates the transcription of alkali-expressed genes (e.g., the alkaline phosphatase and protease genes *palD* and *prtA*, respectively) and also of the penicillin biosynthesis genes *ipnA* (322) and *acvA* (103, 318). The intergenic region between *acvA* and *ipnA* was found to contain four in vitro PACC binding sites designated ipnA1, ipnA2, ipnA3, and ipnA4AB, recognized by a GST-PACC (amino acids 31 to 195) fusion protein (see Fig. 10). The fusion protein was demonstrated to bind to the core consensus GCCARG (322). The mode of DNA recognition by PACC was studied in detail (105).

A mutation analysis of each of these sites by using *ipnA-lacZ* gene fusions revealed that in vivo the binding site ipnA3 was most important for PACC-dependent *ipnA* expression whereas ipnA2 and ipnA4AB were less important, although ipnA2 was bound with highest affinity by PACC in vitro. ipnA1 apparently was not required for PACC-dependent *ipnA* expression (102) (see Fig. 10). As observed for expression of an *ipnA-lacZ* gene fusion (104), expression of an *acvA-uidA* gene fusion was increased in a PacC5 mutant strain (318). Because *pacC5* is an allele which is active irrespective of the ambient pH (322), this suggests that PACC also regulates the expression of *acvA*. This assumption was further supported by the observation that the steady-state levels of *acvA* mRNA were increased at alkaline ambient pH (103). Furthermore, the addition of histidine and valine to the culture medium led to its acidification and to reduced *acvA-uidA* expression. This effect was not observed in

a deletion strain (Δ 183–312) carrying a deletion spanning PACC binding site ipnA3 (at nucleotides 265 to 270) or in the PacC5 mutant strain with a constitutively active PACC protein. Taken together, these data suggest that PACC also regulates the *acvA* expression of *A. nidulans* predominantly from ipnA3 (318). Therefore, an external alkaline pH is sufficient to give rise to considerable transcriptional derepression of steady-state *acvA* and *ipnA* transcript levels, expression of both genes measured by using gene fusions, and penicillin titers.

In addition, at alkaline ambient pH, PACC prevents the transcription of acid-expressed genes (322; reviewed in reference 18). The full-length form of PACC, which predominates at acidic ambient pH or in PalB7 strains grown under acidic or neutral conditions, is not functional (90, 246). It must be specifically proteolyzed to yield the functional (for both positive and negative roles) version containing the N-terminal 40% of the protein (246) (see Fig. 10). The processed form is functional as both the activator and repressor (246; reviewed in reference 18). The *pacC* gene seems to be autogenously regulated, because the *pacC* transcript is strongest at alkaline pH, and the *pacC* promoter contains PACC binding sites (322).

PACC proteolysis occurs in response to a signal provided by the six regulatory *pal* gene products in alkaline environments (246, 322; reviewed in reference 18). Thus, the products of the *palA*, *palB*, *palC*, *palF*, *palH*, and *palI* genes constitute an alkaline ambient pH signal transduction pathway which is required for the conversion of PACC to its functional form (90).

Recently, both the *palA* and *palB* gene have been cloned and sequenced (90, 242). The amino acid sequence of the derived *palA* protein showed similarities to that of a hypothetical protein from the nematode *Caenorhabditis elegans* and also has possible yeast homologs. These data, however, did not allow speculation about the putative function of the protein (242). The predicted *palB* gene product has similarity to the catalytic domain of the calpain family of calcium-activated cysteine proteases. However, PALB does not catalyze the final step of proteolytic processing of PACC (90). The *pal* pathway is thought to introduce a modification of PACC at alkaline pH in wild-type strains, disrupting intramolecular interactions to allow activating proteolysis (246). The type of PACC modification mediated by the *pal* pathway in response to alkaline pH is not known, but an earlier (and more C-terminal) proteolytic cleavage of PACC resulting in susceptibility to further proteolysis might be mediated by PALB (90). Alternatively, it was suggested that PALB might proteolyze one of the other *pal* gene products in a signalling cascade (90).

By cross-hybridization with a nearly full-length *A. nidulans pacC* gene as the probe and a genomic library of *P. chrysogenum* NRRL 1951, the *P. chrysogenum pacC* gene was cloned and sequenced (316). The gene complemented an *A. nidulans* strain carrying a null *pacC* mutation, thus showing that it was functional. The gene is 1,979 bp long and is interrupted by a single intron of 56 bp. The predicted 641-residue protein (M_r 68,681) exhibits most of the features described for the *A. nidulans pacC* protein, including three zinc fingers of the Cys₂His₂ class. Steady-state levels of the *pacC* transcript were elevated at alkaline pH, suggesting that the *P. chrysogenum pacC* transcription was also pH regulated (316).

A fusion protein of GST with amino acids 46 to 154 of *P. chrysogenum* PACC [GST-PACC(46–154)], overexpressed in *E. coli* and purified, bound in vitro to the intergenic region between *P. chrysogenum acvA* and *ipnA*. By computer analysis, seven PACC binding consensus sites (5'-GCCARG-3') were found in the intergenic region. This is consistent with the finding that steady-state *ipnA* mRNA levels were increased at alkaline pH (316).

The upstream region of the *P. chrysogenum* *aacA* gene contains eight binding sites for PACC, whereas that of *A. nidulans* has just one such sequence, suggesting that these genes might be regulated by PACC as well (316).

In addition, several groups have characterized the intergenic region between the *P. chrysogenum* *acvA* and *ipnA* genes by band shift assays. Chu et al. (68) and Feng et al. (110) reported independently that partially purified crude extracts of *P. chrysogenum* bound in vitro to the sequences TGCCAAG and GCCAAGCC, respectively. Although the binding sites were located at different positions in the intergenic region, their degree of identity is striking. Furthermore, the proposed DNA binding proteins of *P. chrysogenum* appear to recognize the same sequence motif which is bound by the PACC proteins of both *A. nidulans* and *P. chrysogenum* (316, 322), which, for *A. nidulans*, has been reported to bind to GCCA(A/G)G (322). Hence, the binding sites identified almost certainly correspond to PACC binding sites (316).

In the intergenic region between *acvA* and *ipnA* of *A. chrysogenum* (230), there are three potential PACC binding sites, making it likely that these genes are also regulated by a PACC homolog. In addition, it will be interesting to analyze whether the genes of the second cephalosporin biosynthesis cluster are regulated by a PACC homolog in *A. chrysogenum*.

pacC genes are not confined to β -lactam-producing fungi, as shown by the recent cloning of the *A. niger* *pacC* gene (202). This gene encodes a three-zinc-finger protein of 667 amino acids (M_r 70,901; gene size of 2192 bp interrupted by two exons of 139 and 52 bp). The gene seems to be pH dependent and thus autogenously regulated. Hence, PACC seems to represent a wide-domain regulator which is involved in the regulation of expression of penicillin biosynthesis genes.

Because the use of glucose or sucrose as the C source leads to acidification of the medium (see, e.g., references 46, 101, and 104), it was conceivable that the glucose/sucrose effect was due to pH regulation (104). This was further supported by the observation that external alkaline pH could bypass the sucrose repression of steady-state *ipnA* transcript levels and penicillin titers. Inclusion of 100 mM Na_2HPO_4 in sucrose broth resulted in an initial pH of 8.0. Northern blot analysis revealed that the *ipnA* mRNA levels under these conditions were completely derepressed (104). Because buffering at alkaline pH with Tris-Cl increased penicillin titers in glucose media as well, this suggested that the increases were not caused by phosphate ions (104, 295). Additional experiments confirmed that alkaline pH is the factor that derepresses penicillin production in 3% sucrose broth (104). Furthermore, analysis of *pacC* mutants revealed that they bypassed carbon regulation of *ipnA* transcript levels, i.e., that *pacC* mutations caused derepression of steady-state levels of the *ipnA* mRNA in sucrose broth despite external acidic pH resulting from sucrose utilization (104). However, neither acidic external pH nor the *palA1*, *palB7*, or *palF5* mutation mimicking the effects of growth at acidic pH prevented C-source derepression (104). Furthermore, the PACC binding sites determined in vitro by the use of a fusion polypeptide containing the PACC DNA binding domain are not located in the *cis*-acting region, which was shown to mediate C-source repression of *ipnA-lacZ* expression (104, 322; see "Carbon source regulation" above). Taken together, these data support the model of Espeso et al. (104) and Tilburn et al. (322) involving independent regulatory mechanisms, one mediating carbon source regulation and another mediating pH regulation through the *pacC*-encoded transcriptional regulator. Since alkaline pH values per se seem to derepress *ipnA* transcription, Espeso et al. (104) proposed that internal alka-

linity represents a physiological signal which triggers penicillin biosynthesis.

However, the use of nonrepressing C sources with respect to *ipnA* steady-state transcript levels, such as 3% lactose, 1% L-arabinose, or 100 mM acetate, as the principal C source resulted in an increase of the external pH. Growth in repressing C sources, such as 3% sucrose, 1% D-glucose or 1% glycerol caused acidification of the media (104). The authors concluded that carbon limitation, either by using less favorable C sources or by reducing the concentration of favorable C sources, results in external alkalization whereas the availability of sufficient amounts of a favorable C source causes external acidification. Thus, carbon and pH regulation normally act in concert although the mechanisms are different (104).

In contrast to the situation in *A. nidulans*, an alkaline ambient pH did not seem to override the negative effect of a repressing C source on *ipnA* transcription in *P. chrysogenum*, because full *ipnA* expression was dependent on carbon source derepression irrespective of the ambient pH (316).

The reason for the pH-mediated regulation of penicillin biosynthesis is unclear. Arst (18) suggested that it might be associated with the observation that β -lactams exhibit increased toxicity on at least some bacterial species at alkaline pH. Furthermore, bacterial competition with fungi may be more intense at alkaline pH (reviewed in reference 18). This agrees with the results of Espeso et al. (104), who concluded that for *A. nidulans*, penicillin biosynthesis in an alkaline environment might be advantageous even if the C source is not limiting, since alkaline pH appears to override C repression. On the other hand, limiting C sources seem to allow derepression of *ipnA* expression at any external pH; i.e., carbon derepression of *ipnA* transcription bypasses the requirement for PACC (104).

Nitrogen Regulation

The effect of the availability of nitrogen source on the penicillin biosynthesis has been discussed for a long time. Sanchez et al. (286) reported the inhibition of penicillin biosynthesis in *P. chrysogenum* by high levels of ammonium. This effect was correlated with low levels of glutamine synthetase. Mycelia grown at low NH_4^+ concentrations accumulated higher concentrations of glutamine in the free pool of amino acids and lower concentrations of glutamate. In contrast, increasing the concentration of NH_4^+ led to a decreased pool of glutamine, an increased glutamate concentration, and poor antibiotic production. It was found that ammonium concentrations $[(\text{NH}_4)_2\text{SO}_4]$ higher than 100 mM also strongly interfered with cephalosporin C production in *A. chrysogenum*. L-Asparagine and L-arginine were better nitrogen sources than ammonium with respect to antibiotic yield (296).

Recently, it was demonstrated that in *P. chrysogenum*, ammonium directly influenced the expression of penicillin biosynthesis genes. By using gene fusions of both penicillin biosynthesis genes *ipnA* and *acvA* with the *E. coli* reporter gene *uidA* and integration of these gene fusions in single copy at the *P. chrysogenum* *niaD* gene locus, it was shown that the expression of both genes was repressed by the addition of 40 mM $(\text{NH}_4)\text{Cl}$ to lactose-grown mycelia (109) (Fig. 5).

Nitrogen metabolite repression is generally regarded as a wide-domain regulatory system, which operates to ensure that a constant nitrogen supply is readily available for growth in response to widely variable or rapidly changing environments. In the absence of favored nitrogen sources, such as ammonium or glutamine, many secondary nitrogen sources, e.g., nitrate,

purines, proteins, and amides, were used. These require the de novo synthesis of permeases and catabolic enzymes, whose expression is highly regulated by the nitrogen regulatory circuit (reviewed in references 58 and 218). In *A. nidulans* and *Neurospora crassa*, global nitrogen repression and derepression is mediated by the major positive control genes *areA* and *nit-2*, respectively (121, 180). Recently, by using a PCR-based approach, the homologous gene of *P. chrysogenum*, *nre*, was cloned and shown to complement Nit-2 mutants of *N. crassa* (132). Each of these three genes encodes regulatory factors with a single Cys-X₂-Cys-X₁₇-Cys-X₂-Cys-type zinc finger, which, in combination with an immediate downstream basic region, constitutes a DNA binding domain. The overall amino acid sequences of these three regulatory proteins show only 30% identity, but they have 98% identity in their DNA binding domains. A similar zinc finger motif has been found in a wide range of eukaryotic organisms including yeast, plants, chickens, mice, and humans. All of these transcription factors recognize the consensus sequence GATA and can be grouped into a GATA protein family (reviewed in reference 218). The optimal binding sites for NIT-2 were found to consist of at least two GATA elements, which can face in the same or opposite directions, with a spacing which can vary from 3 to 30 bp (66).

A protein consisting of 181 amino acid residues of the 835-residue *P. chrysogenum* NRE (132), containing its zinc finger domain, fused to the N terminus of *E. coli* β -Gal was expressed in *E. coli*. Band shift assays with the purified β -Gal-NRE fusion protein showed binding with high affinity to a DNA fragment derived from the intergenic region between *acvA* and *ipnA* of *P. chrysogenum*. Although there are six GATA sequences in the intergenic region, missing-contact experiments with the β -Gal-NRE fusion protein revealed that NRE strongly interacts with a site that contains two of these GATA sequences (133). The same GATA sequences were also strongly bound by *N. crassa* NIT-2 (110). In this binding site, the two GATA core sequences are arranged in a head-to-head fashion and are separated by 27 bp. Therefore, it appears very likely that nitrogen metabolite regulation of penicillin biosynthesis genes is mediated through NRE and that these structural genes for secondary metabolism are regulated as members of a nitrogen control circuit. This suggests that the availability of favored nitrogen sources, and thus good growth conditions, leads to reduced penicillin synthesis by the fungus. However, in vivo studies are clearly needed to relate NRE binding to potential regulatory functions (133).

No evidence for nitrogen-dependent regulation of penicillin biosynthesis in *A. nidulans* has been reported so far. This is also consistent with the observation that the intergenic region between *acvA* and *ipnA* of *A. nidulans* contains only a single GATA motif whereas six GATA sequences are found in the corresponding *P. chrysogenum* region (133). Thus, it seems unlikely that a single GATA motif is bound by an *A. nidulans* GATA factor. However, it cannot be entirely ruled out, because members of the metazoan GATA transcription factor family, each containing two zinc fingers homologous to the single zinc finger motif of NRE, also bind to DNA fragments containing single GATN (where N is A, T, C, or G) motifs (170, 231). Interestingly, the *P. chrysogenum* promoter was shown to respond to nitrogen control when transformed in *A. nidulans*, indicating that the nitrogen-repressing system of *A. nidulans* acted on the heterologous promoter (173). Furthermore, it is worth noting that in the intergenic region of the corresponding *A. chrysogenum* genes (230), there are 15 GATA motifs. It is thus conceivable that these genes are also regulated by a GATA factor.

Amino Acids as Precursors and Mediators of Regulation

Radiochemical tracer studies during the 1940s and 1950s (reviewed in reference 80) revealed that penicillins and cephalosporins are naturally synthesized from the three amino acid precursors L- α -AAA, L-cysteine, and L-valine. Although only cysteine and valine are finally incorporated in the β -lactam nucleus, the requirement for L- α -AAA has long been established (reviewed in reference 86).

Cysteine and valine are ubiquitous, proteinogenic amino acids, whereas L- α -AAA is an intermediate of the L-lysine biosynthetic pathway, which occurs in this form only in higher fungi and euglenids (*Euglena gracilis*) (Fig. 6). The pathway starts from α -ketoglutaric acid and is named according to its key intermediate, α -amino adipate (336; reviewed in references 38 and 331). Hence, L- α -AAA is a branch point between L-lysine and penicillin and cephalosporin biosynthesis (Fig. 6). Genetic data suggested, however, that in *A. nidulans*, L- α -AAA for penicillin biosynthesis is also provided by catabolic conversion of L-lysine by an as yet unidentified pathway. This was concluded from the observation that lysine-auxotrophic mutants defective in the lower part of the pathway leading to the formation of L- α -AAA produced penicillin when L-lysine was added to the medium (330) (Fig. 6).

For *P. chrysogenum*, a similar observation was made (99). A lysine-auxotrophic mutant blocked in a step before L- α -AAA was also able to synthesize penicillin when L-lysine was added to the medium. Additional biochemical studies with [U-¹⁴C]lysine revealed the formation of labeled saccharopine and L- α -AAA. Hence, L- α -AAA can also be obtained for penicillin synthesis by reversal of the last steps of the L-lysine biosynthetic pathway (99) (Fig. 6). In addition, the authors found a high lysine: α -ketoglutarate- ϵ -aminotransferase activity in *P. chrysogenum*, which converts L-lysine into 1-piperidine-6-carboxylic acid. This finding suggests the existence of at least two different pathways involved in the degradation of L-lysine (99). Taken together, these data demonstrate that L- α -AAA can be provided by both the L-lysine biosynthetic pathway and degradation of L-lysine. The contribution of the latter catabolic pathway to penicillin production, however, remains to be shown.

In bacteria, the situation is different, because bacteria and green plants synthesize L-lysine starting from aspartic acid via a different route, designated the diaminopimelic acid pathway. Since L- α -AAA is not an intermediate in this pathway, the question how cephalosporin-producing bacteria obtain the required L- α -AAA was investigated. In *S. clavuligerus*, a specific enzyme activity, lysine ϵ -aminotransferase, was discovered; this enzyme catalyzes the removal of an amino group of L-lysine to give cyclic 1-piperidine-6-carboxylic acid, which is then oxidized to L- α -AAA (205). By this means, β -lactam-producing streptomycetes can form the precursor amino acid L- α -AAA for β -lactam biosynthesis. The gene encoding lysine ϵ -aminotransferase was designated *lat* and cloned from both *S. clavuligerus* (206, 326) and *N. lactamdurans* (76). In both bacteria, it is located within the cephamycin biosynthesis gene cluster (Fig. 3). This supports the finding that it plays a role exclusively in β -lactam biosynthesis (76, 205, 206, 326).

A number of different by-products are commonly encountered during the production of penicillin by *P. chrysogenum*, one of which is the δ -lactam of L- α -AAA, 6-oxo-piperidine-2-carboxylic acid (OPC), formed by cyclization of L- α -AAA. This is the reason why net synthesis of L- α -AAA is required, although the L- α -AAA moiety of IPN can be reused (52, 120, 143, 183, 328) (Fig. 6).

The synthesis of cysteine for β -lactam biosynthesis is an-

other crucial factor in the economics of β -lactam formation. Sulfate assimilation in fungi has been studied primarily in *S. cerevisiae* and *N. crassa* (reviewed in reference 217). Genetic and biochemical analyses have shown that the pathway in the β -lactam-producing *A. nidulans* requires a sulfate permease (*sB*) to transport sulfate into the cell, ATP sulfurylase (*sC*), adenosine-5-phosphosulfate (APS) kinase (*sD*), and 3-phosphoadenosine-5-phosphosulfate (PAPS) reductase (17, 127) (Fig. 7A). Sulfite is reduced to sulfide by the multienzyme complex sulfite reductase, which involves the transfer of six electrons from NADPH to the sulfur atom. The overall consumption of cofactors for the reduction of sulfate to sulfide is 4 mol of NADPH and 2 mol of ATP (Fig. 7A). Thus, the reduction of sulfate to sulfide accounts for a major part of the metabolic costs in the overall biosynthesis of L-cysteine (244). Some of the enzymes involved in β -lactam-producing fungi have been characterized, and the first genes have been cloned (42, 55, 69, 214, 241, 274, 293). The regulation of both cysteine and methionine biosynthesis appears to be complex and awaits further investigations. Recent work conducted on *A. nidulans* and *P. chrysogenum* has indicated an additional mode of regulation by allosteric inhibition, which is absent in yeast (42, 118). This regulation is thought to occur by the binding of PAPS to a pseudosubstrate site located in ATP sulfurylase, thus causing feedback inhibition when excess PAPS is present. At least four regulatory genes control sulfur metabolite repression in *A. nidulans* (241).

Starting from sulfide, three different routes leading to cysteine biosynthesis have been described (reviewed in references 209, 327, and 331) (Fig. 7B). In the direct sulfhydrylation pathway, reduced sulfur is incorporated by *O*-acetylserine sulfhydrylase into *O*-acetyl-L-serine to give cysteine. In a different pathway, transsulfuration, sulfide incorporation is catalyzed by *O*-acetylhomoserine sulfhydrylase. The third possibility is reverse transsulfuration, in which the sulfur of methionine is transferred to cysteine (Fig. 7B). Interestingly, different routes leading to the formation of cysteine are used by the various β -lactam-producing fungi. This is apparently genetically determined. Although all the pathways seem to exist in *A. chrysogenum* (260, 327), the fungus prefers to generate cysteine for optimal cephalosporin C synthesis by conversion of methionine to cysteine via reverse transsulfuration. This finding well agrees with the observation that methionine at certain concentrations has a regulatory function in the cephalosporin biosynthesis of *A. chrysogenum* (see below). In contrast, *P. chrysogenum* and *A. nidulans* synthesize cysteine mainly from direct sulfhydrylation starting from serine (260, 327) (Fig. 7B). The direct sulfhydrylation pathway is energetically more favorable than the transsulfuration pathway (244) (Fig. 7B).

The biosynthesis of valine is closely connected to the biosynthesis of leucine. Valine biosynthesis proceeds by four enzymatic steps with two moles of pyruvate as precursor metabolites (Fig. 8) (reviewed in reference 245). The presence of pools of the three amino acids specific for β -lactam antibiotic biosynthesis (L-cysteine, L-valine, and L- α -AAA), separate from the main pool of each amino acid, has been proposed (144).

Because penicillin and cephalosporin are synthesized from amino acid precursors, it was conceivable that amino acids play a role in the regulation of their biosyntheses. This was supported by the observation that in both *A. nidulans* and *P. chrysogenum* the addition of L-lysine to fermentation medium led to reduced penicillin titers (48, 84). High levels of L-lysine also interfere with cephalosporin production in *A. chrysogenum* (227).

Since L- α -AAA is a branch point between L-lysine and the

penicillin and cephalosporin biosynthetic pathways, L-lysine inhibition of penicillin biosynthesis was suggested to operate at one or more steps of the L-lysine pathway (reviewed in 209) (Fig. 6). This was based on the notion that L-lysine caused feedback inhibition of homocitrate synthase activity in *P. chrysogenum* (88, 119, 200) (Fig. 6). Homocitrate synthase catalyzes the initial reaction of L-lysine biosynthesis. In *P. chrysogenum*, 75% of this activity is in the cytoplasm and 25% is in mitochondria (148). Hence, compartmentalization might be important as well. Whether L-lysine also represses the synthesis of the enzyme is controversial (149, 200).

α -Aminoadipate reductase is also inhibited by L-lysine, and the inhibition occurs at physiological concentrations, i.e., typical cytosolic concentrations of L-lysine (4). It was demonstrated that in *A. nidulans*, another enzyme of the L-lysine biosynthetic pathway required for the synthesis of L- α -AAA, homoaconitase encoded by *lysF*, was reduced in L-lysine-grown mycelia (338). So far, *lysF* is the only gene of the L-lysine biosynthetic pathway cloned from a β -lactam-producing fungus (338). Taken together, it seems very likely that the L- α -AAA pool available for penicillin production is reduced by L-lysine through feedback inhibition and through repression of several L-lysine biosynthesis genes and enzymes (4, 143, 144, 310, 338). When the α -aminoadipate reductase activities (Fig. 6) of three penicillin-producing strains of *P. chrysogenum* were compared, α -aminoadipate (α -AAA) reductase from the lowest penicillin producer was found to be least sensitive to L-lysine inhibition. The activity in vivo of α -AAA reductase from superior penicillin producer strains of *P. chrysogenum* was more strongly inhibited, suggesting that this might explain the ability of high producers to accumulate increased amounts of L- α -AAA and consequently produce more penicillin (196). Furthermore, in *A. nidulans*, L-lysine led to repression of the expression of both *acvA* and *ipnA* gene fusions (48), suggesting, in addition, a more direct effect on the expression of penicillin biosynthesis genes (Fig. 5). Apparently, in the Panlab strain development program, there has been selection against inhibition and/or repression by L-lysine (186).

An important aspect with regard to L-lysine regulation is the compartmentation of the pathway. Biochemical data obtained with *Saccharomyces cerevisiae* suggest that the reactions of the first part of the pathway up to formation of L- α -AAA take place in the mitochondria whereas those of the second part are in the cytosol (37). Hence, L- α -AAA has to leave the mitochondria (Fig. 4). In addition, the major part of intracellular L-lysine is sequestered in the vacuole (38, 143).

It was reported that the addition of D,L-methionine to the *A. chrysogenum* medium (final concentration, 20 mM) led to a three- to fourfold increase in the production of cephalosporin C (96, 335). Early during the investigation of this methionine effect, the question arose whether the role of methionine was solely that of a sulfur donor, because the methionine analog norleucine, lacking a sulfur atom, mimicked the methionine inductive effect (289). Final and conclusive proof that the methionine effect acted at the gene expression level came from Velasco et al. (335). These authors reported that the increased production of cephalosporin C triggered by addition of methionine to the medium was paralleled by increased steady-state levels of the mRNAs of the cephalosporin biosynthesis genes *acvA*, *ipnA*, *cefEF* and, to a slight extent, *cefG*.

Lara et al. (185) reported an inductive effect of L-glutamate on penicillin biosynthesis by *P. chrysogenum*. However, for these experiments, ammonium chloride (8.5 mM) was used as the nitrogen source and was then replaced by L-glutamate (10 mM). With glutamate, cultures produced about five times the amount of penicillin as in the ammonium-grown controls. Re-

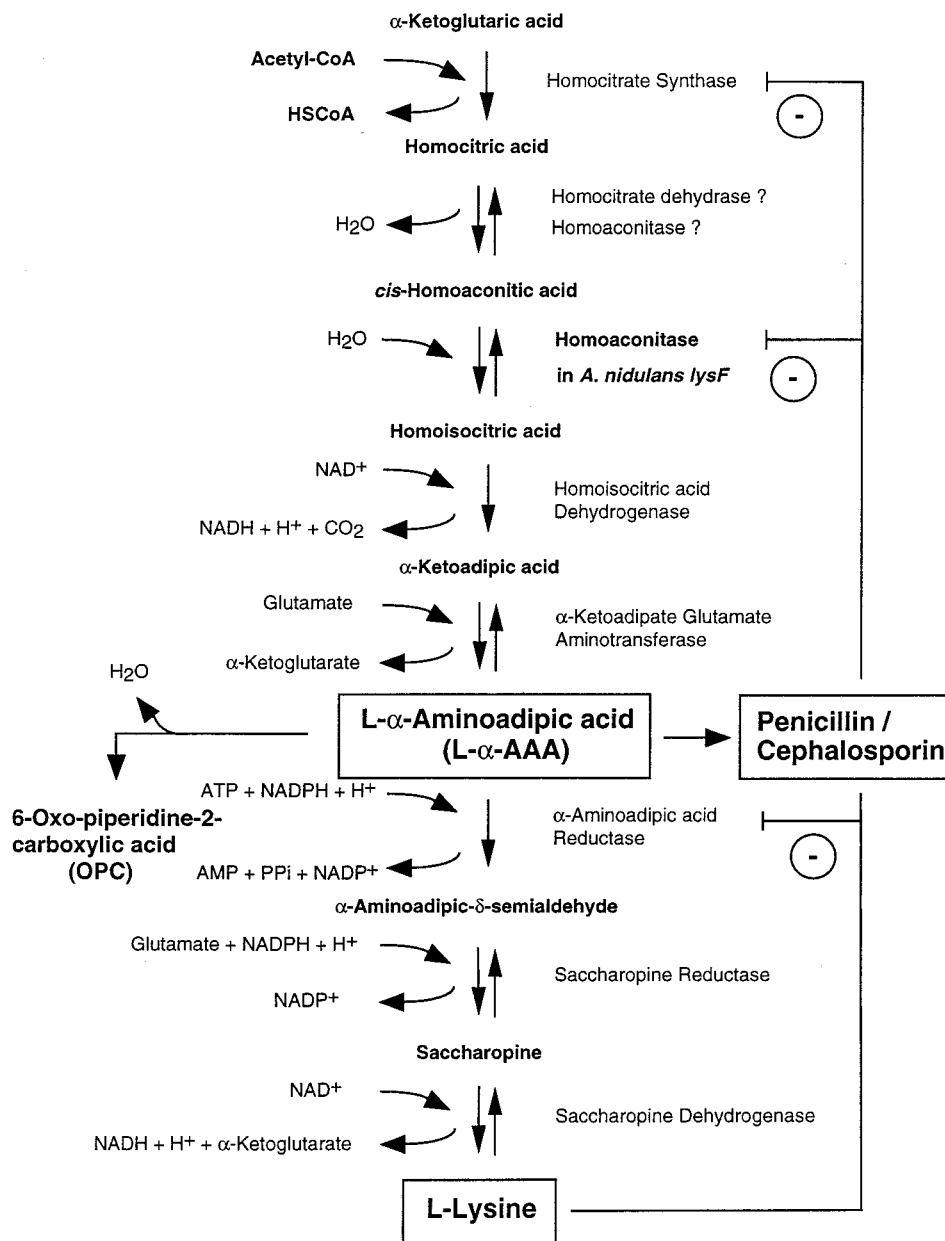


FIG. 6. Lysine biosynthetic pathway (aminoadipate pathway) with the intermediate L- α -AAA. It has not been clarified whether the reaction from homocitric acid to *cis*-homoaconitic acid is catalyzed by homoaconitase, as is the following reaction, or by a specific enzyme designated homocitrate dehydrase (38). Therefore, this reaction is labelled with a question mark. OPC is cyclized L- α -AAA and is found as by-product in the culture broth of penicillin fermentations. The minus signs indicate negative effects of L-lysine on the indicated reactions which were identified from either *P. chrysogenum* or *A. nidulans*. These might be mediated at the transcriptional level or posttranscriptionally. See the text for details.

cently, it was shown that the penicillin biosynthesis in *P. chrysogenum* was reduced when ammonium was used as the nitrogen source. This effect seems to be due, at least in part, to the repression of the penicillin biosynthesis genes *acvA* and *ipnA* by ammonium (109), which seems to be mediated by the nitrogen regulatory protein NRE (133). Therefore, it is conceivable that Lara et al. (185) measured nitrogen repression rather than an inducing effect of glutamate.

It was comprehensively tested whether the addition of different amino acids to the medium affected penicillin biosynthesis by *A. nidulans* (318). Differential effects due to various amino acids on the expression of the penicillin biosynthesis

genes *acvA* and *ipnA* and penicillin production were measured. Interestingly, *acvA* expression seemed to be more subject to regulation by external amino acids than did *ipnA* expression. (i) Some amino acids (e.g., L-threonine, L-aspartate, L-glutamate, and L-cysteine) led to increased *acvA-uidA* expression but had no major effect on *ipnA-lacZ* expression. (ii) The amino acids L-methionine (at concentrations greater than 10 mM), L-leucine, L-isoleucine, L-phenylalanine, L-valine, L-histidine, and L-lysine led to repression of both *acvA-uidA* and *ipnA-lacZ* expression, which was dependent on the concentration used. (iii) The amino acids L-tyrosine, L-tryptophan, L-proline, and L- α -AAA had no major effect on *acvA-uidA* expression but led

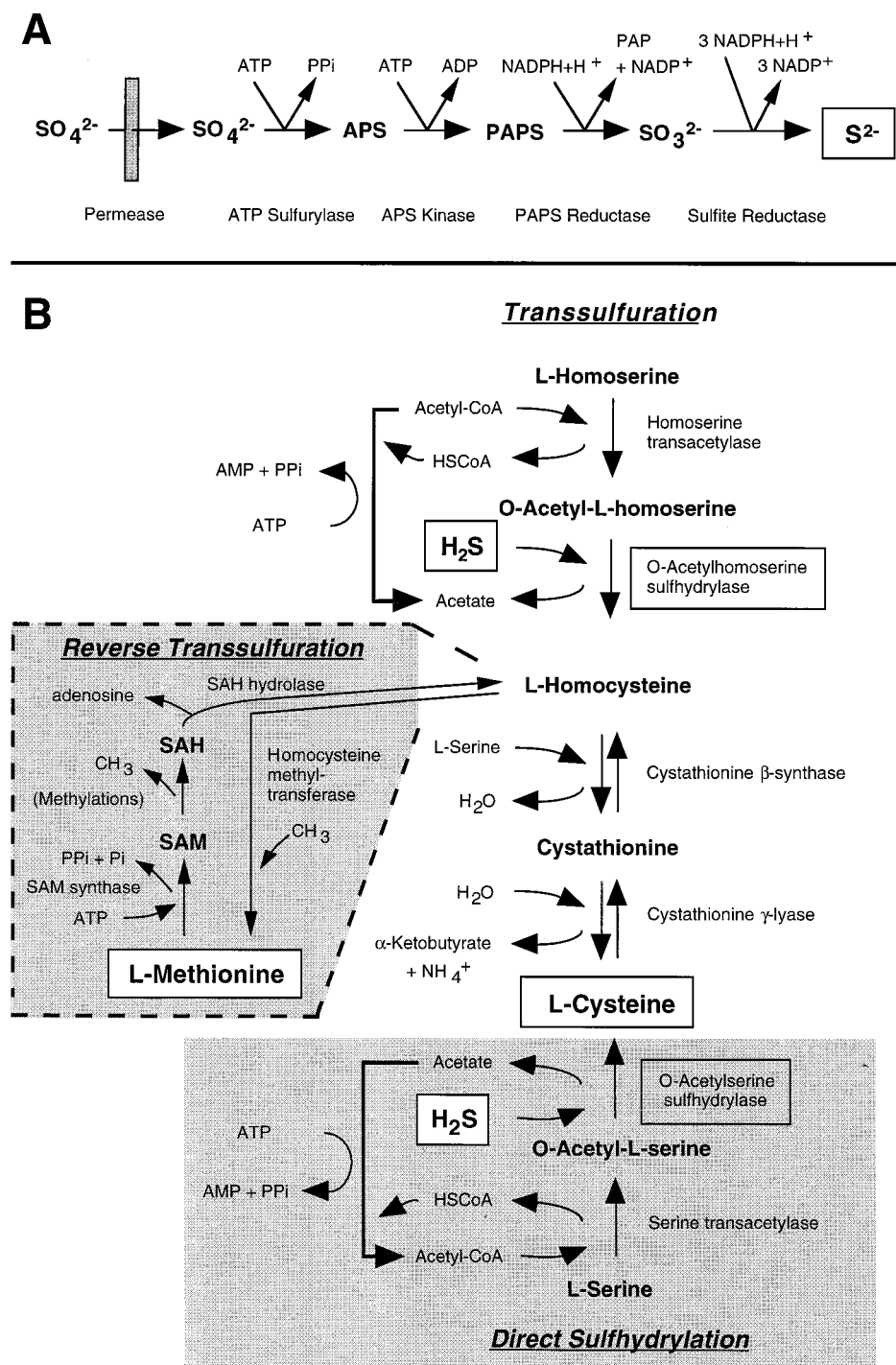


FIG. 7. Pathways leading to the synthesis of L-cysteine. (A) Assimilation of sulfate. For reduction of sulfate to sulfide, several enzymes are required. Sulfate is transported into the cytoplasm by a specific permease, encoded by the *sB* gene in *A. nidulans*. See the text for details. (B) Starting from sulfide, three different routes leading to the formation of cysteine are used by the various β -lactam-producing fungi. They are designated transsulfuration, direct sulfhydrylation (grey background), and reverse transsulfuration (grey background bounded by broken lines). Although all the pathways seem to exist in *A. chrysogenum*, the fungus prefers to generate cysteine for optimal cephalosporin C synthesis by conversion of methionine to cysteine via reverse transsulfuration. In contrast, *P. chrysogenum* and *A. nidulans* synthesize cysteine mainly by direct sulfhydrylation starting from serine. See the text for details. Abbreviations: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

to repression of *ipnA-lacZ* expression. (iv) Two amino acids, L-serine and L-arginine, did not show any effect on the expression of either gene fusion at any of the concentrations tested.

L-Amino acids with a major negative effect on the expression of *acvA-uidA* and *ipnA-lacZ* gene fusions, i.e., histidine, valine,

lysine, and methionine (only at concentrations greater than 10 mM), led to decreased penicillin titers and a decreased ambient pH during cultivation of the fungus. An analysis of deletion clones lacking binding sites of the pH-dependent transcriptional factor PACC (104, 246, 322) in the intergenic region

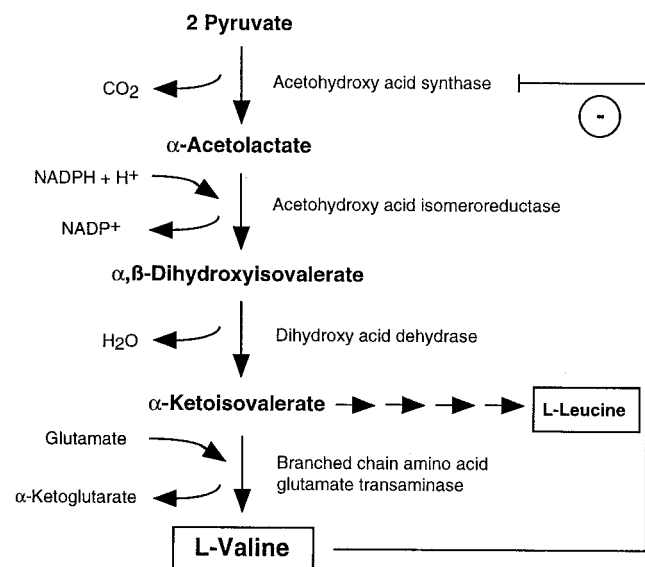


FIG. 8. Biosynthetic pathway of L-valine. The minus sign indicates a negative effect of L-valine on the indicated reaction, as determined in *P. chrysogenum* and *A. chrysogenum*. See the text for details.

between the *acvA-uidA* and *ipnA-lacZ* gene fusions and in a PacC5 mutant strain suggested that the negative effects of L-histidine and L-valine on *acvA-uidA* expression were due to reduced activation by PACC under the acidic conditions caused by these amino acids (see Fig. 10). These data also implied that PACC regulates the expression of *acvA*, predominantly through PACC binding site *ipnA3* (see Fig. 10). The repressing effect caused by L-lysine and L-methionine on *acvA*, however, was even enhanced in one of the deletion clones and the *pacC5* mutant strain, suggesting that these amino acids act independently of PACC by exerting effects on gene expression by unknown mechanisms (Fig. 5). For L-methionine, the analysis of deletion clones indicated that a major *cis*-acting DNA element responsible for its effect on *acvA-uidA* expression is located between nucleotides 433 and 569 of the intergenic region. For L-lysine, no DNA region which could be involved has yet been identified. The effects of L-methionine and L-lysine in the medium are mediated by regulatory mechanisms, which appear to differ even between these two amino acids (318). It is interesting that L-lysine and L-methionine are closely related to the precursor amino acids L- α -AAA and L-cysteine, respectively.

Velasco et al. (335) noticed several consensus CANNTG sequences in the intergenic region of the *A. chrysogenum* cephalosporin biosynthesis genes *acvA* and *ipnA* whose transcription was increased after addition of D,L-methionine. Members of the basic region-helix-loop-helix (bHLH) protein family recognize such a consensus motif. There are many bHLH proteins which are involved in the regulation of a number of genes. These proteins can bind as homo- or heterodimers to DNA (190). Some of these transcription factors are involved in the transcriptional control of the sulfur network in *S. cerevisiae* (22, 59, 229, 321). Thus, the authors suggested that a member of the bHLH proteins might mediate these effects. By analogy, it is conceivable that the effect of L-methionine on the corresponding *A. nidulans* genes is due to the action of a similar regulator. However, since there is no conserved CANNTG consensus motif in the region spanned by nucleotides 433 to 569 of the intergenic region between *acvA* and *ipnA*, it remains to be

elucidated how the effect of methionine is mediated at the molecular level.

Influence of Phosphate and Oxygen

Excess phosphate was found to exert a negative effect on cephalosporin production in *A. chrysogenum* W53253 (181). The basis of this effect is still a matter of debate, and the molecular mechanism is not understood. It was suggested that phosphate increased the consumption rate of glucose, thus enhancing glucose repression (181), whereas resting-cell experiments indicated that in the absence of glucose, phosphate itself decreased the overall flux to formation of cephalosporin C (213). This was further supported by Zhang et al. (348), who found a direct negative effect of phosphate on formation of several enzymes (ACVS, IPNS, and DAOC synthetase/DAC hydroxylase) of the cephalosporin biosynthesis pathway. It was suggested that phosphate acted on IPNS and DAOC synthetase/DAC hydroxylase by complexing the iron needed for enzyme activity, because the inhibition of these two enzymes could be reversed by adding more ferrous iron (197). However, even inhibition of ACVS, which does not require ferrous iron for activity, was reversed by the addition of Fe^{2+} (349). Therefore, the cause of the inhibitory effect of phosphate is not yet fully understood.

The availability of oxygen is clearly important for penicillin production. Good aeration of mycelia with oxygen is a prerequisite for high β -lactam titers (139; reviewed in reference 317). Since several enzymes, such as IPNS and DAOC synthetase/DAC hydroxylase, require oxygen for their activity, it is conceivable that this is the reason for the oxygen requirement. The importance of oxygen is also supported by the possibility of increasing cephalosporin production genetically by introducing a bacterial oxygen binding protein into *A. chrysogenum* (89; see "Applications" below). However, there is a contradictory report which shows that reduction of oxygen led to increased *acvA* and *ipnA* expression of *P. chrysogenum*, possibly as part of a stress response (273).

The CCAAT Box Binding Protein Complex PENR1

Since several regulatory circuits affect the regulation of penicillin biosynthesis and presumably more remain to be discovered, a variety of *cis*-acting DNA elements and regulatory factors can be expected to be involved. Some of these have been found by analysis of mutants (*pacC* and *pal*) or by physiological and biochemical analyses (*nre*). For a comprehensive search of additional *cis*-acting DNA elements, a deletion analysis of the *A. nidulans ipnA* gene promoter (257) and a moving-window analysis of the intergenic region between the *A. nidulans acvA* and *ipnA* genes (319) were carried out. In the latter, the effects of deletions in the intergenic region were analyzed for the expression of both genes, *acvA* and *ipnA*, simultaneously.

Based on the results of this moving-window analysis and together with band shift and methyl interference assays, a CCAAT-containing DNA motif (box I) located 409 bp upstream of the ATG initiation codon of the *A. nidulans acvA* gene, which is bound by a protein complex designated PENR1 (penicillin regulator 1), was identified (319) (see Fig. 10). This CCAAT box I is of major importance for the regulation of both genes, since a 4-bp deletion within this site ($\Delta\text{CCA-G}$) led to an eightfold increase of *acvA* expression and simultaneously to a reduction of *ipnA* expression to about 30% of the original level (319). Furthermore, scanning of the *A. nidulans aatA* promoter region by band shift assays, using protein fractions eluted with different KCl concentrations in the buffer from a

heparin-agarose column, led to the identification of an additional CCAAT-containing DNA element (box II) located about 250 bp upstream of the transcriptional start sites of *aatA*. All biochemical data suggested that it was specifically bound by the same PENR1 regulatory protein complex. Substitution of the CCAAT core sequence by GATCC led to a fourfold reduction of expression of an *aatA-lacZ* gene fusion (195), indicating that the identified binding site was functional in vivo and positively influenced *aatA* expression (see Fig. 10).

Because PENR1 bound to a CCAAT-containing DNA sequence, this suggested that it belongs to the class of CCAAT binding proteins (195, 319). CCAAT boxes are present in the promoters of many eukaryotic genes. They are bound by distinct transcription factors, which may explain the variety of functions that have been attributed to the CCAAT sequences in the modulation of transcript levels in eukaryotic cells (see, e.g., references 32, 225, 236, 261, 272, and 287; reviewed in references 158 and 233). Several CCAAT binding factors have been found to bind to their target sequence as a complex of heterologous subunits, or heteromers (see, e.g., references 67, 134, and 272). For the time being, it is unknown how many different CCAAT binding proteins exist in filamentous fungi.

Some genes of *A. nidulans* have also been reported to contain functional CCAAT sequences in their promoter regions. Band shift experiments and deletion analysis showed that a CCAAT binding factor (AnCF) is involved in the regulation of the acetamidase gene *amdS*, which is required for the use of acetamide as the nitrogen and C source (189, 332). In addition, the intergenic region of the bidirectionally transcribed genes *lamA* and *lamB* (needed for utilization of lactams) and the promoter region of *gata* (γ -amino butyric acid transaminase) contain CCAAT boxes which were bound by DNA binding factors (278). For the *gata* CCAAT sequence, this also most probably seems to be AnCF (252). An additional CCAAT binding factor (AnCP) of *A. nidulans* was proposed (163, 240) which bound in vitro to the Taka-amylase gene promoter of *A. oryzae*. AnCP was later found to be most probably identical to AnCF (see below). Finally, deletion of a CCAAT box in the promoter of the developmentally regulated *yA* gene of *A. nidulans* led to elevated expression of a *yA-lacZ* gene fusion (15).

So far, the only CCAAT box binding factor that has been characterized in detail both genetically and biochemically in lower eukaryotes is the *S. cerevisiae* HAP complex, which consists of at least four subunits: HAP2, HAP3, and HAP5, which form a heterotrimeric complex that is essential for DNA binding, and HAP4, which is an acidic protein that acts as the transcriptional activation domain. Mutations in either of the *hap* genes led to the inability of yeast cells to grow on nonfermentable C sources such as lactate. It was shown that the HAP complex regulates, for example, the iso-1-cytochrome *c* (*CYC1*) gene required for respiration (see, e.g., reference 226; reviewed in reference 128).

Recently, Papagiannopoulos et al. (252) reported the cloning of a gene from *A. nidulans* designated *hapC* that exhibits significant similarity to the *S. cerevisiae* HAP3 gene. In a *hapC* deletion strain (Δ hapC), the activity of the CCAAT binding factor AnCF, which is present in wild-type strains and was detected by the binding of AnCF to CCAAT boxes present in the promoter regions of the genes *gata* and *amdS*, was missing. In addition, the Δ hapC strain hardly grew on acetamide as the sole nitrogen and C source, indicating that HAPC in fact plays a role in regulating *amdS* expression. This was confirmed by analyzing an *amdS-lacZ* gene fusion, which showed reduced expression in a Δ hapC background. Hence, it seems very likely that HAPC is a member of the proposed AnCF complex that binds at least to the CCAAT motif in the *gata* and *amdS*

promoters. Furthermore, band shift and supershift experiments with polyclonal antibodies against HAPC revealed that AnCP also contains the HAPC protein (164).

For the CCAAT-binding complex PENR1, its binding consensus motif was determined by band shift assays as RRCC AAT(C/A)RCR (320). The deviation of a single base from the consensus motif reduced the percentage of bound DNA to at least 40% (for some bases even to 2 to 10%). Since the surrounding bases of the CCAAT site in the *amdS* promoter (AGCCAATCACC) match the PENR1 binding consensus motif except for the last cytosine, it was conceivable that HAPC is also part of PENR1 and, consequently, that AnCF and PENR1 share at least some protein components. Partial purification of PENR1 and Western blotting with anti-HAPC antibodies indicated that HAPC was highly enriched during purification of PENR1. Furthermore, band shift assays with partially purified crude extracts of a Δ hapC mutant strain revealed that in such crude extracts no PENR1 activity was detectable. Immunoprecipitation and supershift experiments with anti-HAPC antibodies provided evidence that HAPC is a component of the PENR1 complex (193). PENR1 thus represents a HAP-like transcriptional complex, as does AnCF.

Deletion or mutagenesis of the PENR1 binding sites had opposite effects; the expression of *acvA* was increased eightfold (319), while the expression of *ipnA* (319) and *aatA* (195) was reduced (see Fig. 10). Formally, the CCAAT box I mediated a negative effect on *acvA*, but a positive effect on *ipnA*, and box II a positive effect on *aatA* expression (see Fig. 10). Consistent with data obtained by deletion of the PENR1 binding site, expression of an *ipnA-lacZ* gene fusion in a Δ hapC background was reduced to about 15% compared with its expression in the wild type. A similar effect was observed for the expression of an *aatA-lacZ* gene fusion, which was reduced to 10% in the Δ hapC strain. Hence, PENR1 is of major importance as a positively acting factor of *ipnA* and *aatA* expression. Interestingly, the expression of *ipnA* and *aatA* gene fusions in the Δ hapC strains was even more reduced than in strains which had a deletion or substitution of the relevant CCAAT boxes. This might suggest that in addition to the identified CCAAT boxes I and II, other CCAAT boxes are important for the activity of PENR1 in vivo. Both in the intergenic region between *acvA* and *ipnA* and in the *aatA* promoter, there are additional CCAAT boxes. However, the analysis of the whole *aatA* promoter by band shift assays did not reveal another site apart from box II which was bound by PENR1 in vitro (195). This finding does not entirely exclude, however, that another CCAAT site which is upstream of box II might be relevant to a certain extent in vivo, although it can only be bound with low affinity by PENR1 in vitro.

Penicillin titers were reduced in a Δ hapC background as well, but only by about 30%. In addition, expression of an *acvA-uidA* gene fusion was hardly affected by the Δ hapC mutation (193). The minor effect of lack of PENR1 on penicillin production is consistent with the view that *acvA* expression is rate limiting in *A. nidulans* wild-type strains (165). Whereas overexpression of the *acvA* gene led to a massively increased penicillin production (165), simultaneous overexpression of both *ipnA* and *aatA* had little effect on penicillin production (112). In contrast, decreased expression of *ipnA* and *aatA* even by a factor of 5, as observed in the Δ hapC strain, results in a reduction of penicillin production of only about 30% when the expression of the *acvA* gene is only marginally affected.

The observation that *acvA* expression was less strongly affected in a Δ hapC strain was unexpected because it was shown that specific deletion of the four nucleotides CCA plus the 3' G of the PENR1 binding site between *acvA* and *ipnA* (box I)

resulted in a strong increase of *acvA* expression (319). It thus appears likely that in addition to PENR1, a repressor protein binds closely to or overlaps the PENR1 binding site, which would explain why the PENR1 binding site exhibits a repressing effect on *acvA* expression in the wild type. Consistent with this view, lack of PENR1 binding in the Δ hapC mutant did not prevent binding of this putative repressor protein and hence *acvA-uidA* expression was not increased. Deletion of the PENR1 binding site, however, prevented binding of both PENR1 and the repressor, causing the phenotype of increased *acvA* expression. However, the existence of such a putative repressor is hypothetical and remains to be shown experimentally. Proteins binding overlapping or adjacent to CCAAT boxes have been previously noted for several CCAAT-containing sequences in vertebrates and fungi (32, 97, 332). It would thus be very interesting to elucidate whether this also applies to the PENR1 binding sites (193). In summary, PENR1 represents a HAP-like complex which shares core proteins, at least HAPC, with AnCF. Because PENR1 does not bind to all CCAAT-containing sequences in vitro (195, 319) and, in addition, it is difficult to imagine that the *amdS* gene is regulated by the identical complex to that regulating the penicillin biosynthesis genes (and thus belongs to the same regulatory circuit), it seems likely that PENR1 differs from AnCF in an accessory protein(s). *A. nidulans* genes designated *hapE* (253) and *hapB* (192), exhibiting similarity to the *S. cerevisiae* *hap5* and *hap2* genes, respectively, have been cloned and sequenced. The availability of these genes will help to elucidate both PENR1 and its relation to the AnCF complex of *A. nidulans* and, in addition, to search for further proteins that may be part of the PENR1 complex.

As a result of PENR1 binding, a DNA fragment spanning the CCAAT box I between *acvA* and *ipnA* was distorted in vitro by more than 94°. The center of the flexure was predicted to be near or at the CCAAT sequence (193). A similar observation was made for the murine NF-Y complex, which is analogous to the *S. cerevisiae* HAP complex (281). Binding of NF-Y resulted in distortion of DNA to a degree dependent on the composition of neighboring sequences, varying between 62 and 82°. The center of the bend was also the CCAAT box (281).

The physiological function of HAP-like regulatory factors in lower eukaryotes remains obscure. In yeast, the HAP complex activates the expression of genes whose products are required for respiration. Hence, Hap mutants are not able to grow on nonfermentable carbon sources (see, e.g., reference 226; reviewed in reference 128). Because *A. nidulans*, however, is an aerobic fungus, *S. cerevisiae* may not be a good model for the role of HAP-like complexes in aerobic eukaryotes. Furthermore, the lack of a functional HAP complex (Δ hapC strain) is not lethal for *A. nidulans* (252). In addition, the HAP-like PENR1 complex in *A. nidulans* regulates secondary-metabolism genes (penicillin biosynthesis genes). Hence, it will be interesting to elucidate whether this particular function of a HAP-like complex requires as yet unknown accessory proteins, or, alternatively, which mechanism is involved in allowing a HAP-like complex to regulate certain sets of genes.

It is interesting that deletion of 4 bp of the PENR1 binding site led to increased *acvA* expression and reduced *ipnA* expression (319). It has not been clarified whether this increase of *acvA* and decrease of *ipnA* expression is of physiological relevance. It is tempting to speculate, however, that under certain physiological conditions an increase of *acvA* expression and a simultaneous reduction in the expression of *ipnA* and *aataA* could lead to larger cellular amounts of ACV. The tripeptide could have, apart from being the precursor of β -lactams, additional functions, e.g., in amino acid transport, as suggested by

Del Carmen Mateos and Sanchez (83). ACV is structurally very similar to glutathione (GSH), consisting of the tripeptide γ -(L-glutamyl)-L-cysteinyl-glycine. Both ACV and GSH contain the free thiol group of the cysteine residue. They also have one unusual peptide bond between the δ -carbon atom of L- α -AAA and the γ -carbon atom of glutamate and the cysteine residue in ACV and GSH, respectively. In higher eukaryotes, GSH is a part of the γ -glutamyl cycle, which, among other functions, serves to transport extracellular amino acids across the plasma membrane (228). Experiments with resting cells of *P. chrysogenum* implied that the ACV tripeptide might be important for the uptake of glutamine from the medium (83). Consistent with this hypothesis is the observation that early during a fermentation run, both ACV tripeptide and the δ -lactam OPC (cyclized L- α -AAA) are found in the fermentation broth (52, 159). OPC appears very similar to 5-oxoproline, the cyclized product of the γ -glutamyl moiety of GSH, produced during GSH-dependent amino acid transport. Under certain physiological conditions, it might thus be advantageous for the fungus to produce the tripeptide first (e.g., for amino acid transport). Then, when binding of PENR1 and/or a putative repressor causes the expression of *acvA* to be reduced and simultaneously the expression of *ipnA* and *aataA* to be increased, the tripeptide is channelled towards penicillin. In addition, ACV might serve the function of fixing cysteine, which, at high levels, is toxic. Alternatively, the presence of a repressor could provide an additional possibility to fine-tune *acvA* expression. These assumptions, however, are speculative and need to be tested in the future.

Since HAPC is part of PENR1, it seems very likely that the PENR1 complex is conserved among the industrially important β -lactam-producing fungi. This assumption is supported by the observation that DNA fragments spanning the corresponding intergenic regions between *acvA* and *ipnA* of *P. chrysogenum* and *A. chrysogenum* and the promoter region of the *P. chrysogenum* *aataA* gene were able to dilute the complexes of the corresponding *A. nidulans* probes and *A. nidulans* PENR1 protein (195, 319). Computer analysis showed that DNA elements with a high degree of sequence identity to the *A. nidulans* PENR1 site reside within the intergenic regions of both *P. chrysogenum* and *A. chrysogenum* and the *aataA* promoter region of *P. chrysogenum*. These sites could be potential targets of homologous PENR1 complexes in *P. chrysogenum* and *A. chrysogenum*.

trans-Acting Mutations Affecting the Expression of Penicillin Biosynthesis Genes

The molecular approaches so far described led to the identification of DNA binding proteins involved in the regulation of penicillin biosynthesis genes. These regulatory proteins seem to represent wide-domain regulators (PACC and NRE). For the HAP-like complex PENR1, this cannot be predicted with certainty at the moment, although it seems likely. However, by analogy to the regulation of biosynthesis of secondary metabolites in streptomycetes (reviewed in reference 39), it can be expected that pathway specific regulatory genes exist.

To identify both such putative genes and regulatory genes not belonging to the family of DNA binding proteins (such as, e.g., coactivator proteins or members of a signal transduction cascade), a combination of classical and molecular techniques was independently used by Pérez-Esteban et al. (256) and Brakhage and Van den Brulle (50). The aim was the isolation of mutants of *A. nidulans* carrying mutations which are specifically involved in *trans* in the regulation of expression of the penicillin biosynthesis genes. The rationale of the approach

can be summarized as follows. *A. nidulans* strains carrying *ipnA-lacZ* gene fusions integrated in double-copy at the chromosomal *argB* gene locus were used (Fig. 9). On minimal agar plates supplemented with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) as an indicator for β -galactosidase activity, colonies of such a strain stained blue, indicative of *ipnA-lacZ* expression. After mutagenesis, colonies which did not produce a blue color or produced only a faint blue color were isolated. Cells of such colonies most probably carried mutations affecting the expression of *ipnA-lacZ* gene fusions in *trans*. The identification of *cis*-acting mutations, i.e., in the *ipnA* promoter or the *lacZ* gene, seemed to be unlikely, because such mutations would probably not be detected due to the second gene fusion located on the chromosome (Fig. 9).

By using this approach, mutants carrying recessive mutations were identified, and the genes were designated *prg* (penicillin regulation) (50) and *npeE1* (impaired in penicillin biosynthesis) (256). In penicillin production medium, the mutants exhibited only 5 to 50% of the level of *ipnA-lacZ* expression and produced only 10 to 30% of the amount of penicillin compared with the wild-type strain. It was demonstrated that mutants Prg-1 (*prgA1*) and Prg-6 (*prgB1*) also differed in *acvA-uidA* expression levels from the wild type and, by Western blot analysis, that these mutants contained reduced amounts of the *ipnA* gene product, i.e., IPNS (50). Segregation analysis indicated that in both mutants the Prg phenotype resulted from mutation of a single gene. Two different complementation groups were identified, which were designated *prgA1* (mutant strain Prg1) and *prgB1* (mutant strain Prg6). However, the specific activity of the *aata* gene product was essentially the same in the *prgA1* and *prgB1* mutants as in the wild type, implying that the last step of the penicillin biosynthetic pathway might not be affected, at least to the same extent, by these *trans*-acting mutations.

Genetic analysis showed that the *npeE1* gene is located on linkage group IV (256). To date, it has not been clarified whether *npeE1* differs from *prgA1* and *prgB1*. The results obtained by genetic and biochemical analyses indicated that the mutants isolated most probably carry mutations in positively acting regulatory genes, which in the case of *prgA1* and *prgB1* specifically affect both *acvA* and *ipnA* and in the case of *npeE1* affect at least *ipnA* expression (Fig. 10). Cloning of the corresponding genes will show to which kind of putative regulatory genes these mutations correspond and to which regulatory circuits they belong.

Using nitrosoguanidine, Cantoral et al. (60) isolated nine mutants of *P. chrysogenum* Wis54-1255 impaired in penicillin production. Biochemical and genetic analysis suggested that two of these (mutants Npe2 and Npe3) carry mutations in regulatory genes affecting the expression of the entire penicillin biosynthesis gene cluster.

Posttranscriptional Regulation

Discrepancies observed between the expression of structural genes and enzyme specific activities of the corresponding proteins suggested that besides transcriptional regulation, posttranscriptional regulation of penicillin biosynthesis genes occurs (47, 194, 346).

Mycelia of *A. nidulans* grown with glucose showed reduced IAT specific activities compared with those grown with lactose. When mycelia were first grown with glucose for 12 h, washed to remove remaining glucose, and subsequently transferred to medium with lactose, the IAT specific activities of mycelia rapidly increased, and reached even higher values after 24 h than did control cultures incubated with lactose only (194).

This suggested that the effect of glucose on IAT specific activity was reversible. When cultures were first grown with lactose and then transferred to medium containing glucose, the IAT specific activity decreased compared with values measured in mycelia which were further incubated with lactose. In contrast, the expression of an *aata-lacZ* gene fusion was only marginally reduced in glucose-grown mycelia compared with lactose-grown mycelia. Taken together, these results suggested that the effect of glucose on IAT specific activity was posttranscriptionally mediated (194).

For the *A. chrysogenum* ACVS, some posttranslational regulation was also observed with respect to its reduced activity measured in crude extracts obtained from glucose-grown mycelia (346, 349) (see "Carbon source regulation" above).

REGULATION OF β -LACTAM BIOSYNTHESIS IN FUNGAL PRODUCTION STRAINS

Apart from the academic interest in elucidating the molecular regulation of biosynthesis of secondary metabolites in lower eukaryotes, there is a strong interest from an industrial point of view because β -lactam compounds are still the most widely sold antibiotics in the world's antibiotic market (reviewed in reference 211). Hence, it is desirable to analyze highly producing production strains which are highly mutated and have been derived from several different strain development programs. This will help to elucidate the molecular basis of deregulation and of the high production and also any remaining bottlenecks.

Nowadays, industrial penicillin and cephalosporin production is carried out mainly with *P. chrysogenum* and *A. chrysogenum*, respectively. Most of these strains have been produced by mutagenesis followed by screening or selection. In 1972, the initial Panlabs Inc. *P. chrysogenum* strain made 20,000 U of penicillin per ml in 7 days (an activity equivalent to 12 mg of pure penicillin G, Na salt, per ml [317]). In 1990, the improved strain made 70,000 U per ml in 7 days. Penicillin titers in industry in 1993 were as high as 100,000 U per ml (reviewed in references 211 and 219).

Commercial production of penicillin began in 1941 with *P. notatum* NRRL 1249-B21 (Northern Regional Research Laboratory, Peoria, Ill.) in surface culture fermentation. The search for superior production strains led to the isolation of *P. chrysogenum* NRRL 1951 (271). This strain became the ancestor of strains developed at the University of Wisconsin, which resulted in a family of strains (Wis strains) producing ever higher titers of penicillin. Strains descended from members of this series continue to be isolated by pharmaceutical companies (243).

Two important genetic features of *P. chrysogenum* production strains have been identified: (i) amplification of structural genes and (ii) their massively increased steady-state mRNA levels.

Slot blot and densitometry analysis of genomic DNA from the highly producing strain *P. chrysogenum* BW1890 (Beecham Pharmaceuticals; derived from the Wisconsin series of strains [reviewed in reference 244]) probed with the *ipnA* gene indicated that between 8 and 16 copies are present in that strain (304). Furthermore, Southern analysis demonstrated that the entire 39-kb insert of cosmid pCX3.2 carrying all penicillin biosynthesis structural genes (305) is amplified in this high-titer production strain. Northern blot analysis established that the steady-state level of *ipnA* mRNA in strain BW1890 was 32- to 64-fold that of NRRL 1951, an increase too great to be due to the amplification alone (304). Independently, Barredo et al. (33) showed that in the *P. chrysogenum* high-titer-producing

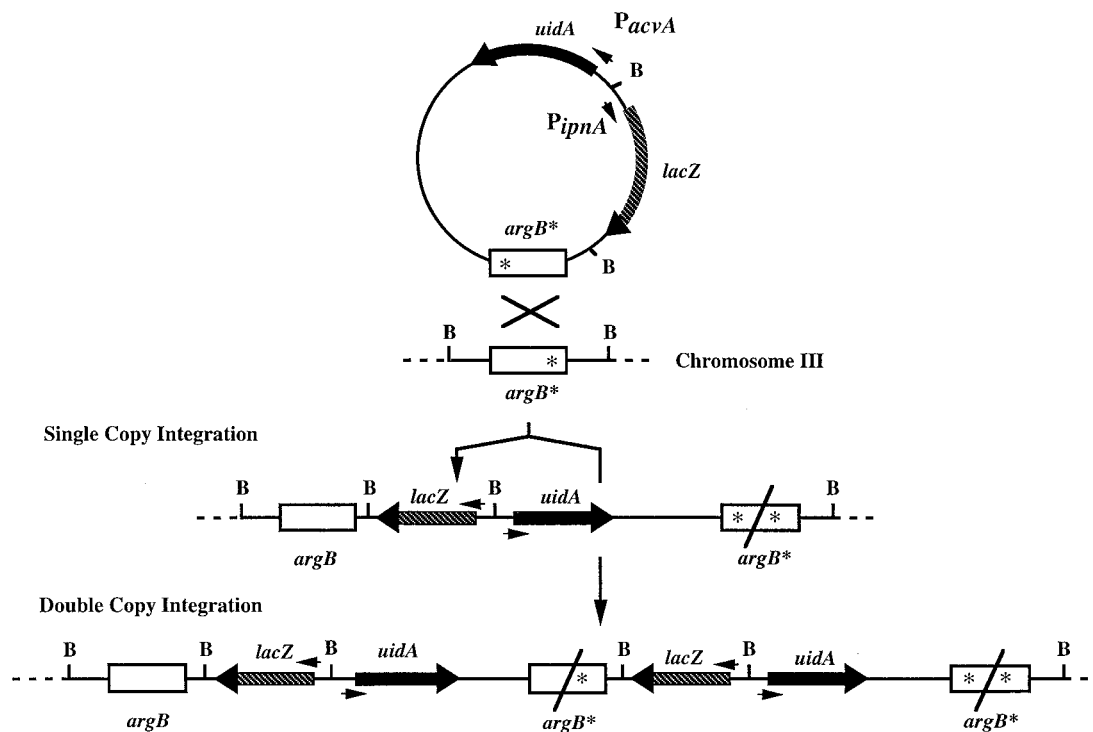


FIG. 9. Diagrammatic representation of possible homologous recombination events of a plasmid (pAXB4A) carrying *acvA-uidA* and *ipnA-lacZ* gene fusions integrated in single or double copy at the chromosomal *argB* gene locus of *A. nidulans*. The restriction map around the *argB* locus with and without integrated plasmid is shown. By using an appropriate probe, transformants carrying the plasmid integrated in double copy were identified by Southern blot analysis (46, 50). Essentially the same system with an *ipnA-lacZ* gene fusion only was independently used by Pérez-Esteban et al. (256) to identify the *npeE1* mutation. Abbreviations: *argB**, mutated *argB* allele which can complement the chromosomal *argB2* mutation by homologous recombination; B, *Bam*HI restriction site; *lacZ*, *E. coli* β -galactosidase gene; *PacvA*, promoter region of the *acvA* gene; *PipnA*, promoter region of the *ipnA* gene; *uidA*, *E. coli* β -glucuronidase gene. Modified from reference 50 with permission of the publisher.

strains P-2 (a highly producing strain from Nippon Kayaku Co. which was used as the ancestor of many strains in the Panlab series [reviewed in reference 244]) and AS-P-78 (old production strain used by Antibióticos [reviewed in reference 244]), the copy numbers of penicillin biosynthesis genes are approximately nine- and sixfold, respectively.

Fierro et al. (114) showed that in the high-titer *P. chrysogenum* strains E1 and AS-P-78, the amplifications are organized in tandem repeats. A conserved TTTACA hexanucleotide sequence was suggested to be involved in their generation. This TTTACA sequence borders the 106.5-kb penicillin biosynthesis gene cluster in the wild-type strain NRRL 1951 and also in *P. notatum* ATCC 9478 (Fleming's isolate). Furthermore, three non-penicillin-producing *P. chrysogenum* mutants were independently isolated. It was shown that in all of these mutants deletion of the penicillin biosynthesis gene cluster had occurred at a specific site within the conserved hexanucleotide sequence (116). The authors suggested that this site may represent a hot spot for site-specific recombination after mutation with nitrosoguanidine, with the process possibly being part of a fungal SOS system similar to that found in *E. coli* (114, 116).

This analysis was extended to the characterization of other members of the SmithKline Beecham Pharmaceuticals strain improvement series (243). It was found that the amplicon was 57.5 kb long in these strains. Sequence analysis of the promoter regions of the *acvA*, *ipnA*, and *aatA* genes in the high titer strain BW1901 and comparison with wild-type sequences have not identified any potentially titer-enhancing mutations. Furthermore, cDNA screening has not identified any further transcribed elements within the coamplified region apart from

those derived from the structural penicillin biosynthesis genes. The homogeneity of hybridization patterns and the identification and analysis of a single-copy revertant have shown that the amplification is of a direct tandem nature. It was proposed that whether or not the initial recombination event was homologous, once duplication had been achieved, a large stretch of homologous DNA was available for misalignment and hence further amplification or indeed deletion (243).

It appears that in both the SmithKline Beecham strain series and strain E1 (114), the amplified regions are bounded by the same nonamplified *Bam*HI DNA fragments, although the amplicons are apparently not identical (243). Taken together, these data indicated the presence of recombinogenic regions flanking the penicillin biosynthesis gene cluster (114, 243).

Since sequence analysis of promoter regions has shown that no mutations have been generated within the promoter regions of the penicillin biosynthesis structural genes (243), these data suggest that the amplification of gene clusters may be accompanied by changes in *trans*-acting factors. This is also consistent with the observation that in the high-titer-producing *P. chrysogenum* strain BW1890 the increase in the steady-state level of *ipnA* mRNA was too great to be due to the amplification alone (304). However, data obtained with several production strains indicate that penicillin titers were not proportionally increased with copy number. Hence, in these strains the effect of other limiting factors, such as primary metabolic pathways or, in these cases, *trans*-acting regulatory factors, must be alleviated before the full potential of the extra copies is realized (243). Therefore, it will be of considerable interest to compare regulatory genes already found in both *A. nidulans* and *P. chryso-*

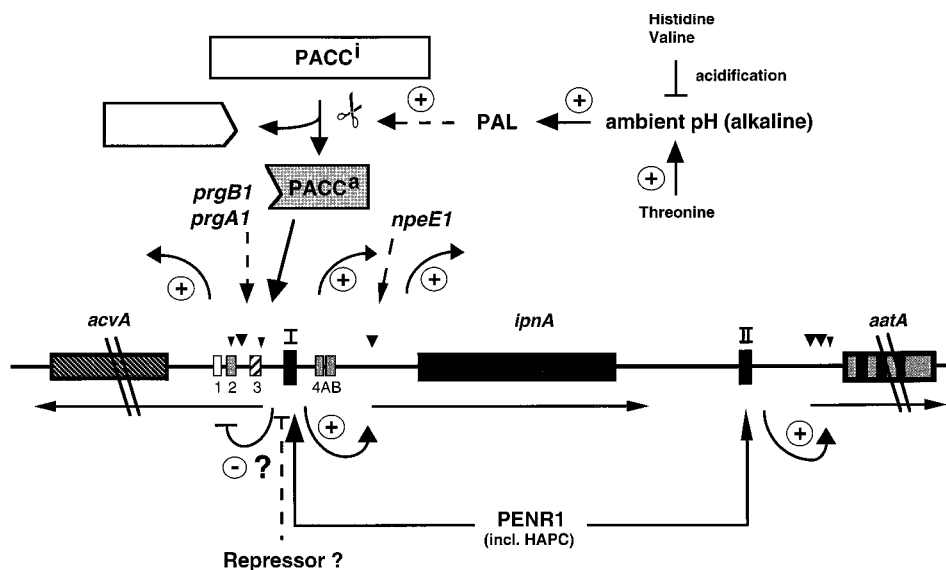


FIG. 10. Regulatory genes involved in the regulation of the penicillin biosynthesis genes of *A. nidulans*. Addition of the indicated amino acids to the medium results in the indicated effects on gene expression (318). Some of these effects are mediated by the ambient pH, most probably via *pal* genes and the central regulatory protein PACC encoded by the *pacC* gene (318). PACCⁱ symbolizes the inactive form, which is proteolytically cleaved upon the pH signal (alkalinity) into PACC^a, i.e., the active version of PACC (246, 322). The two identified PENR1 binding sites containing CCAAT sequences are marked with black boxes and roman numerals (I and II). One of the protein components of the PENR1 complex is HAPC (in parentheses below PENR1). The four PACC binding sites bound in vitro by PACC (322) are designated 1 (*ipnA1*), 2 (*ipnA2*), 3 (*ipnA3*), and 4AB (*ipnA4AB*) (102) and are boxed in the intergenic region between *acvA* and *ipnA*. PACC sites that were found to be functional in vivo for expression of *ipnA-lacZ* and/or *acvA-uidA* are indicated by hatched boxes. Site 3 seems to be of major importance for both *ipnA-lacZ* (102) and *acvA-uidA* (318) expression. Large and small arrows indicate major and minor transcription start sites, respectively. Names printed in italics indicate mutations which formally represent *trans*-acting genes with positive effects on the expression of the indicated genes. The existence of the repressor is hypothetical. Only two putative regulatory proteins have been described so far in *P. chrysogenum*. PACC most probably mediates the effect of pH on *ipnA* transcription (316), and NRE mediates the nitrogen regulation of *acvA* and *ipnA* (109, 132) (Fig. 5).

genum between *P. chrysogenum* wild-type and production strains.

Furthermore, during the later stages of the fermentation in high-penicillin-producing strain BW1890, a high level of IPNS specific activity was maintained which was not due to increased transcription (304). The stability of IPNS might have increased, because results with *A. nidulans* imply that IPNS might be inactivated during its catalytic reaction (47).

The IAT specific activity was reduced in cells of *A. nidulans* and wild-type *P. chrysogenum* NRRL 1951 grown in medium containing glucose (46) but not in glucose-grown cells of the *P. chrysogenum* production strain AS-P-78 (277). The loss of the effect of glucose on IAT might be important for *P. chrysogenum* production strains.

In contrast to the gene amplification of structural genes reported in *P. chrysogenum* production strains, the β -lactam biosynthesis genes seem to be present in single copy in the cephalosporin C production strain *A. chrysogenum* LU4-79-6 (299, 301). Kück et al. (179) showed that *A. chrysogenum* DSM produces a considerably larger amount of *ipnA* transcript and of cephalosporin C (10-fold larger amount) than does strain ATCC 140553. Hence, as already demonstrated for penicillin production, increased transcription and consequently expression of biosynthesis genes seems to be an important feature of *A. chrysogenum* production strains.

Several high-cephalosporin C-producing *A. chrysogenum* strains contained chromosome rearrangements which partly involve the chromosome carrying the *acvA* and *ipnA* gene (300, 302, 337). However, for the time being, it is unclear whether rearrangements have an impact on the cephalosporin biosynthesis in production strains.

There are certainly numerous other mutations involved which lead to a high-producer phenotype. These also concern

factors such as deregulation of enzymes involved in amino acid biosynthesis pathways and hence the amount of precursor amino acids produced. It was shown, for instance, that improved *P. chrysogenum* penicillin production strains have reduced catabolism of L- α -AAA and flux of L- α -AAA to L-lysine (143). In addition, α -aminoacidate reductase activity from the lowest penicillin producer of *P. chrysogenum* was least sensitive to L-lysine inhibition whereas the enzyme activity of superior penicillin producer strains was more strongly inhibited. This might explain the ability of highly producing strains to accumulate increased amounts of L- α -AAA, and consequently more penicillin (196) (see "Amino acids as precursors and mediators of regulation" above). Goulden and Chattaway (126) found that a high-producing *P. chrysogenum* strain had twice the acetohydroxy acid synthase activity of an ancestral strain and that the enzyme in the superior strain was deregulated to valine feedback inhibition (Fig. 8). A similar observation was reported for an *A. chrysogenum* highly producing mutant, in which acetohydroxy acid synthase was found to be partially desensitized to valine feedback inhibition (224). These alterations seem to be required to provide the large amounts of valine needed for penicillin production. Furthermore, transport of the intermediates of penicillin biosynthesis between organelles and the number of organelles can be predicted to also be important for penicillin production strains.

APPLICATIONS

The increasing knowledge of the molecular genetics of β -lactam biosynthesis has opened up new possibilities to rationally improve β -lactam production strains and to engineer new biosynthetic pathways. This leads to the question, however, whether an improvement of productivity is still possible. Cep-

allosporin C production by *A. chrysogenum* is well below the productivity reached with *P. chrysogenum* for penicillin. Therefore, much effort is needed to increase cephalosporin C production. For penicillin, several theoretical models have been established based on the available experimental data (summarized in reference 244). By using detailed stoichiometric models, the theoretical yield was calculated to be 0.47 to 0.50 mol of penicillin per mol of glucose (136, 160). Up to now, the maximum theoretical yields calculated are 8 to 10 times higher than the overall yields observed in fed-batch cultures, and there is therefore a considerable potential for further improvement of the process. However, it seems unlikely that the maximum theoretical yields can be reached in a real process, since the penicillin biosynthesis is indirectly coupled to other cellular reactions. Therefore, taking this into account, it was estimated that it should still be possible to improve the current yields by a factor of 4 to 5, which would have a dramatic effect on the economy of the process (244).

Several molecular strategies have been followed to improve or alter β -lactam production. (i) β -Lactam biosynthesis genes have been overexpressed by introducing additional copies or by using strong promoters. Manipulation of regulatory genes has not been reported yet because the regulatory genes have only recently been identified. (ii) β -Lactam biosynthetic pathways have been metabolically engineered by expression of heterologous genes, e.g., production of cephalosporin precursors in *P. chrysogenum*. (iii) The increasing knowledge about peptide synthetase genes, such as those for ACVS enzymes, has been used to produce novel compounds by genetic engineering (combinatorial biology [208, 312], as also discussed for polyketide synthetases [137]).

Increase of Expression of β -Lactam Biosynthesis Genes

Kennedy and Turner (165) demonstrated that *acvA* expression in *A. nidulans* is rate limiting for penicillin production. The weak *acvA* gene promoter was replaced by the strong inducible ethanol dehydrogenase promoter (*alcAp*). The expression level of *alcAp* was determined by using a strain in which the reporter gene, *lacZ*, was under the control of *alcAp*. It was found to be up to 100 times higher than that from the *acvA* promoter when induced under fermentation conditions with the artificial inducer cyclopentanone (10 mM). Penicillin yields were increased by as much as 30-fold when the *acvA* gene was overexpressed by induction of the *alcA* promoter. Glucose, which strongly represses transcription from *alcAp*, also repressed penicillin biosynthesis in the overproducing strain.

Fernández-Cañón and Peñalva (112) overexpressed both the *ipnA* and *aataA* genes of *A. nidulans*. For this purpose, they placed the promoters of these genes under the control of the *alcA* promoter. Transcriptional induction of the chimeric genes resulted in 10-fold-higher levels of *ipnA* or *aataA* transcripts than of those resulting from transcription of the corresponding endogenous genes. This increase caused a 40-fold rise in IPNS activity or an 8-fold rise in IAT activity. Despite this rise in enzyme levels, forced expression of the *ipnA* gene resulted in only a modest increase in levels of exported penicillin (increase by about 25%), whereas forced expression of the *aataA* gene even reduced penicillin production (a decrease of about 10 to 30%), showing that neither of these enzymes is rate limiting for penicillin biosynthesis. These findings agree well with those of Kennedy and Turner (165) that the expression of *acvA* is rate limiting for penicillin production in *A. nidulans*. They are also consistent with the observation that the massive reduction of *ipnA* and *aataA* expression caused by deletion of the *hapC* gene,

whose product is part of PENR1, resulted in only a moderate reduction of the penicillin titer because *acvA* expression was only marginally, if at all, affected by deletion of the *hapC* gene (193).

Furthermore, a genomic version of the *alcAp-aataA* fusion in which the *aataA* gene is interrupted by three introns was inducible by threonine (an inducer of *alcAp*) to a lesser extent (as determined by both *aataA* mRNA levels and IAT enzyme levels) than was the corresponding cDNA version. This finding suggests that processing of the introns present in the primary transcript may limit *aataA* overexpression (112).

Consistent with data obtained with *A. nidulans*, attempts to increase cephalosporin C yields in *A. chrysogenum* 394-4 (improved cephalosporin C production strain) by inserting multiple copies of the *ipnA* gene were unsuccessful (301). However, Veenstra et al. (334) transformed *P. chrysogenum* Wis 54-1255 (an early strain of the strain improvement series) with a DNA fragment containing the *P. chrysogenum* Wis 54-1255 *ipnA* and *aataA* genes. Some of the resulting transformants produced up to 40% more penicillin V, indicating that in this particular *P. chrysogenum* strain the products of *aataA*, *ipnA*, or both can be used to improve penicillin production.

Skatrud et al. (301) reported a 15% increase in cephalosporin C production in an industrial strain of *A. chrysogenum* (394-4) by transformation of the strain with an extra copy of the DAOC synthetase/DAC hydroxylase gene (*cefEF*) (to give strain LU4-97-6) (Fig. 2). However, when the experiments were carried out, it was not known that *cefG* encoding acetyl-CoA:DAC acetyltransferase is closely linked to *cefEF* (Fig. 3) and was also present on the plasmid used for transformation. Then it was demonstrated that transformants of wild-type *A. chrysogenum*, carrying additional copies of the *cefG* gene only, showed a direct relationship between *cefG* copy number, *cefG* mRNA levels, and cephalosporin C titers, suggesting that this enzyme might be a rate-limiting step in cephalosporin C production (219, 221). Hence, the effect of the *cefEF* gene alone in increasing cephalosporin C production remains unclear.

Taken together, these data show that in a wild-type strain of *A. nidulans*, expression of biosynthesis genes, at least of the *acvA* gene, is rate limiting. It remains to be elucidated, however, whether this holds for penicillin and cephalosporin production strains. Nevertheless, the expression level of biosynthesis genes, as shown for the cephalosporin C production, is of great importance.

Genetic Engineering of β -Lactam Biosynthetic Pathways

Cloned β -lactam biosynthesis genes can already be used for rational improvement of β -lactam production. DAOC and cephalosporin C can be enzymatically deacylated to form 7-aminodeacetoxycephalosporanic acid (7-ADCA) and 7-aminocephalosporanic acid (7-ACA), respectively; these are important intermediates in the manufacturing of oral cephalosporin antibiotics (Fig. 11). Medically important oral cephalosporins, e.g., cephalexin and cephadrine, are synthesized by derivatizing the 7-amino group of 7-ADCA or 7-ACA with appropriate side chain moieties (56). Although cephalosporins are superior antibiotics compared to penicillins, their production is limited because the process for producing 7-ADCA and 7-ACA is complex. Removal of the natural D- α -aminoadipyl side chain from cephalosporins is inefficient (82, 222). Hence, there have been efforts in both directions: isolation of superior enzymes to remove the side chain directly and development of alternative biosynthetic routes to 7-ACA and/or 7-ADCA (82, 222).

At Eli Lilly & Co., clinically important cephalosporins were

apparently produced by isolating penicillin G from *P. chrysogenum* and by chemical ring expansion of this natural product, which requires protection and sulfoxidation reactions followed by chemical deprotection (62, 264). The resulting deacetoxycephalosporin G is enzymatically deacylated to yield 7-ADCA (61, 62). Cantwell et al. (62) proposed a biological process in which penicillin V was used instead of penicillin G because *P. chrysogenum* strains that produce large amounts of penicillin V are available. For expansion of the thiazolidine ring of penicillin V to give a dihydrothiazine ring characteristic of ceph-3-ems, a modified penicillin N expandase from *S. clavuligerus* with a changed substrate specificity would be required. This enzyme should recognize and convert penicillin V to deacetoxycephalosporin V by ring expansion better than the wild-type enzyme does. In the last step, deacetoxycephalosporin V is enzymatically hydrolyzed to 7-ADCA with a penicillin amidase (62). Although this has apparently not been achieved experimentally yet, initial experiments showed that it is possible to express an *S. clavuligerus* penicillin N expandase gene (*cefE*) in *P. chrysogenum*. The *cefEF* gene of *A. chrysogenum* would not be useful for this purpose because it also encodes the hydroxylase function (61) (Table 4). 3'-hydroxylated cephalosporins, however, are not useful for the manufacturing of 7-ADCA (62). Based on this, Crawford et al. (82) expressed the *S. clavuligerus* *cefE* (expandase) or *A. chrysogenum* *cefEF* (expandase-hydroxylase) gene, with and without the acetyltransferase gene (*cefG*), in *P. chrysogenum* by fusing the genes to the *P. chrysogenum* *ipnA* or the β -tubulin gene promoter (Fig. 11). Feeding of such transformants with adipic acid led to the production of cephalosporins having an adipyl side chain (Fig. 11). This proved that adipyl-6-APA is a substrate for either enzyme in vivo. Transformants expressing *cefE* (expandase) produced adipyl-7-ADCA, whereas transformants expressing *cefEF* (expandase-hydroxylase) produced both adipyl-7-ADCA and adipyl-7-aminodeacetylcephalosporanic acid (adipyl-7-ADAC). Transformants expressing *cefEF* and *cefG* (acetyltransferase) produced adipyl-7-ADCA, adipyl-7-ADAC, and adipyl-7-ACA. The adipyl side chain of these cephalosporins was easily removed with a *Pseudomonas*-derived glutaryl amidase to yield the cephalosporin intermediates (Fig. 11). Hence, by these measures, an important step in cephalosporin manufacturing could be improved (82).

Cephalosporin C biosynthesis is regulated by the oxygen content of the medium (139). The overall rate of cephalosporin C synthesis is severely reduced under conditions of low oxygen. Reduction of the oxygen supply leads to the accumulation of penicillin N (Fig. 2). The mechanism of oxygen control is not understood. It may be connected to the oxygen-requiring reactions involved in cephalosporin biosynthesis (see "Influence of phosphate and oxygen" above). DeModena et al. (89) improved aerobic metabolism in *A. chrysogenum* by using the oxygen binding heme protein hemoglobin from the bacterium *Vitreoscilla*. Its structural gene (*VHb*) was fused with the strong constitutive TR1 promoter from *Trichoderma reesei*. After transformation of *A. chrysogenum* C10, the protein was produced as demonstrated by Western blotting. Several transformants produced significantly higher yields of cephalosporin C than did control strains in batch culture experiments.

Generation of Novel Compounds by Genetic Engineering of Peptide Synthetases

In general, peptide synthetases such as ACVS enzymes possess a highly conserved domain structure. The arrangement of these domains determines the number and order of the amino acid constituents of the peptide product (reviewed in reference

169). Hence, the shuffling of domains, i.e., reprogramming of the protein template, should result in the synthesis of new peptides exhibiting novel amino acid orders. This was achieved by Stachelhaus et al. (312), who exchanged domain-coding regions of bacterial and fungal origin. The authors developed a system allowing targeted substitution of amino acid-activating domains within the *srfA* operon, which encodes the protein templates for the synthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. By this method, new hybrid genes were produced whose products showed altered amino acid specificities. The genes were expressed in *B. subtilis* and led to the production of novel peptides, indicating that the rational design of bioactive peptides by recombinatorial biology is feasible (reviewed in reference 208).

CONCLUSIONS AND FUTURE DIRECTIONS

Research on the regulation of biosyntheses of β -lactam antibiotics is heading toward the elucidation of (i) further regulatory circuits involved, (ii) inducing and repressing signals, (iii) signal transduction pathways (missing links between regulatory circuits and regulatory genes), (iv) additional regulators (transcriptional factors, coactivators, and corepressors), and (v) the mode of action of these regulatory proteins. Understanding these aspects will also help to elucidate the physiological and ecological functions of β -lactams for the producing fungi. The application of this knowledge will contribute not only to a further increase of β -lactam production and the production of novel related compounds but also to the identification of new β -lactam-producing organisms by genetic means. For some of these aspects, a considerable increase in our knowledge has already been accumulated; the following discussion summarizes the underlying results and their future directions.

β -Lactam biosynthesis genes are transcribed from specific promoters and regulated transcriptionally and very probably posttranscriptionally. Transcription of biosynthesis genes is subject to sophisticated control by nutritional factors (e.g., glucose, nitrogen which is putatively mediated by NRE in *P. chrysogenum*), the amino acids lysine and methionine, and ambient pH. It can be assumed that there are other regulatory circuits which remain to be discovered.

With the exception of PACC, which mediates pH-dependent transcription, the regulatory factors responsible for the other regulatory circuits remain unknown. For the repressing effects on *A. nidulans* gene expression mediated by both L-lysine and L-methionine, their mechanistic basis (signals, regulatory factors) is still obscure. The same applies to the effect of methionine in *A. chrysogenum*. The effects are regulated at the transcriptional level, and for the methionine effect in *A. nidulans* a region probably containing the *cis*-acting DNA element responsible could be located. Nevertheless, the underlying mechanisms are unknown and even seem to differ for L-lysine and L-methionine.

For both penicillin and cephalosporin biosynthesis, the carbon source is important. Glucose and sucrose lead to reduced β -lactam production. It was shown that some β -lactam biosynthesis genes are repressed by these C sources. Interestingly, the repressor CREA, which mediates C-source-dependent repression of some *A. nidulans* genes, is not involved. This implies that C-source regulation of β -lactam biosynthesis genes does not follow the general mechanism of C-source regulation.

Nitrogen regulation of the penicillin biosynthesis genes in *P. chrysogenum* is most probably due to the action of NRE, which appears to represent a wide-domain nitrogen regulator homologous to the *A. nidulans* AREA factor. However, the involve-

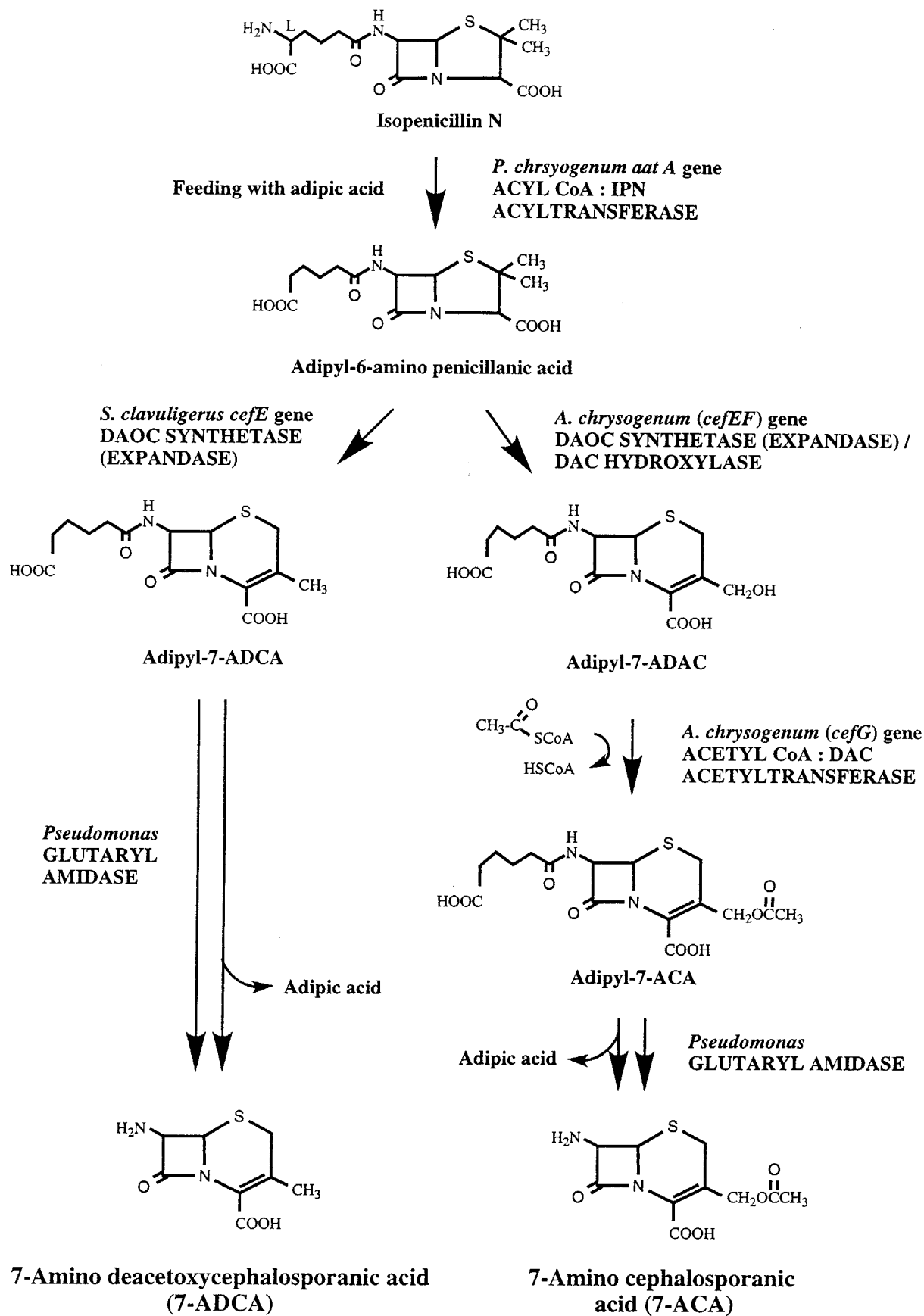


FIG. 11. Production of cephalosporin intermediates via production of adipyl-cephalosporins by using recombinant strains of *P. chrysogenum* (82). The single arrows indicate reactions of the recombinant *P. chrysogenum* strains, and the double arrows indicate contact of the culture filtrate with immobilized *Pseudomonas* amidase. Modified from reference 82 with permission of the publisher.

ment of NRE in nitrogen regulation of *P. chrysogenum* penicillin biosynthesis genes remains to be confirmed by in vivo studies.

Major questions concern the mechanistic basis of the regulatory circuits identified, and in the case of the HAP-like regulatory complex PENR1, the physiological signals activating this factor.

Much of our knowledge about the molecular regulation of penicillin biosynthesis genes has resulted from work on the fungal pH regulatory system (104, 246, 295, 322). This regulation is particularly interesting because it involves the proteolytic activation of a transcription factor by unknown molecular signals which are triggered by an alkaline ambient pH. Furthermore, several components of the regulation have already been elucidated, i.e., the regulatory circuit including the signal (alkaline ambient pH) and the mechanistic basis (transcriptional factor PACC). Based on the identified and available regulatory *pal* mutants, the tools are available to investigate the underlying signal transduction pathway.

It is interesting that although the overall patterns of penicillin production are similar in *A. nidulans* and *P. chrysogenum*, it appears that differences exist. Suarez and Peñalva (316) proposed that in *P. chrysogenum* the genes seem to be regulated more strictly than in *A. nidulans*, e.g., with respect to pH regulation but also with respect to C source and nitrogen regulation. The authors proposed that penicillin production might be more important for the competitiveness and survival of *P. chrysogenum*. This seems to be supported by the fact that wild-type *P. chrysogenum* isolates generally produce more penicillin than *A. nidulans*. However, this cannot be concluded with certainty because for screening of penicillin-producing isolates, only strains which showed increased penicillin production were selected. Thus, this might represent the (pathological) exception rather than the usual production level of *P. chrysogenum*.

Another question of particular interest is whether the genes are regulated by pathway-specific or wide-domain regulators. This question cannot be answered yet. However, the participation of PACC and possibly NRE, which are also present in non-penicillin-producing fungi, and the fact that the genes are expressed in the non- β -lactam producer *A. niger* (194, 306) indicate that they are regulated, at least in part, by wide-domain regulatory genes. For the HAP-like complex PENR1, this has not been confirmed yet, although it seems likely. PENR1 consists of subunits which are also involved in the regulation of other than β -lactam biosynthesis genes, such as *amdS*. However, since not all components of PENR1 have been determined yet, it is unclear whether this particular complex only regulates penicillin biosynthesis. Since PENR1 binds to only certain CCAAT sequences with high affinity in vitro, it appears possible that PENR1 regulates only some genes. The physiological function of HAP-like regulatory complexes in lower eukaryotes remains obscure. In *S. cerevisiae*, the HAP complex activates the expression of genes whose products are required for respiration. *S. cerevisiae* predominantly ferments carbon sources. Therefore, *hap* mutants are not able to grow on nonfermentable carbon sources (reviewed in reference 128). However, because *A. nidulans* is an aerobic fungus, *S. cerevisiae* may not be a good model for the role of HAP-like complexes in aerobic eukaryotes. Hence, further characterization of the regulatory complexes PENR1 and AnCF, which at least have common protein components, even if they are not identical, is needed to elucidate the structure and function of these complexes. If PENR1 regulates other genes than the penicillin biosynthesis genes, such as *amdS* and *gatA*, it will be very interesting to elucidate why these genes belong to a com-

mon regulatory circuit. Candidates for pathway-specific genes, however, might be found among the recessive mutations identified (*prgA1*, *prgB1*, and *npeE1*) in *A. nidulans* and by the mutations carried by the *P. chrysogenum* mutants Npe2 and Npe3, which seem to specifically affect the expression of penicillin biosynthesis genes.

In addition to positively acting regulators, all data are consistent with the involvement of negatively acting regulatory factors. A repressor of *acvA* expression has been proposed in *A. nidulans* which binds close to or overlaps the PENR1-binding site (box I) (Fig. 10).

Also, because fungi are eukaryotes, it can be expected that the regulation of β -lactam genes differs from that in prokaryotes. This view is consistent with the finding that all regulators identified in fungal β -lactam biosynthesis which are summarized here have no counterpart in prokaryotes and vice versa. A regulatory gene (*ccaR*) of both cephamycin and clavulanic acid biosynthesis was found to be located in the cephamycin biosynthesis gene cluster of *S. clavuligerus* (Fig. 3). This gene shows high similarity to regulatory genes involved in the biosynthesis of other secondary metabolites in streptomycetes, such as actinorhodin biosynthesis (258). Furthermore, some fungal regulators might be part of larger complexes, as proposed for the HAP-like transcriptional complex PENR1. This would also have some effect on strain improvement programs, because it might be required to overproduce all components of such complexes in order to obtain overexpression of β -lactam biosynthesis genes and thus overproduction of β -lactams.

So far, only alkaline ambient pH has been identified as a physiological signal which triggers penicillin biosynthesis (104). It appears that the expression of genes is to a certain extent intrinsically constitutive, although this cannot be concluded with absolute certainty. It might well be that signals that have not yet been identified, such as cell density, which was proposed to stimulate β -lactam production in streptomycetes (285), are important. In addition, despite significant levels of gene expression in minimal medium, there is only little penicillin production, even when sufficient amounts of side chain precursor molecules are fed. This might imply that not only is the production of penicillin of relevance, but also the production of the ACV and/or IPN precursor molecule, which could serve additional physiological functions, is relevant. Nevertheless, the presence of a basal gene expression level implies that the presence of penicillin and/or precursor molecules is advantageous to the fungus under different physiological conditions, although their production consumes energy. This basal gene expression level seems to be modulated in response to the different environmental and/or endogenous stimuli; e.g., growth is maximal in the presence of rapidly metabolizable carbon sources whereas gene expression and production of penicillin is reduced. Possibly, fungi do not need to produce large amounts of antibiotics to compete with bacteria when their growth rate is maximal as a result of the availability of good C and N sources. In general, β -lactam production seems to be optimal when the growth of fungi is suboptimal. Therefore, the elucidation of regulatory genes might also contribute to our understanding of such questions related to the physiological function of these compounds for the producing fungus.

It is unclear whether β -lactams serve another function such as defending the habitat against bacteria. It was suggested that the ACV tripeptide could have a function in amino acid transport or could help to fix cysteine, which is toxic at high concentrations. Alternatively, because the last step of penicillin biosynthesis occurs in microbodies and can lead to the attachment of aromatic rings to IPN, this might represent a mechanism of detoxifying these compounds and excreting them to the

environment. There are numerous such compounds in the environment of penicillin-producing fungi, e.g., derived from the degradation of lignin. All of these theories, however, have yet to be proved experimentally.

Since gene expression has a major effect on the production of β -lactam antibiotics in production processes, the knowledge of the molecular regulation of their biosynthesis genes can be expected to be of great importance for rational strain improvement programs. This is particularly relevant because theoretical calculations based on flux analyses have indicated that there is still an enormous potential to increase β -lactam productivity. Therefore, overexpression of regulatory genes that positively affect the expression of both the β -lactam biosynthesis genes and precursor amino acid biosynthesis genes will be attempted and negatively acting regulatory genes will be inactivated.

Although considerable progress has been made in the understanding of the molecular regulation of penicillin and cephalosporin biosynthesis in fungi, our picture is far from complete.

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